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ACTIVATION OF PLASMA BY CONTACT WITH GLASS: EVIDENCE FOR A COMMON REACTION WHICH RELEASES PLASMA KININ AND INITIATES COAGULATION

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(*Received* 3 *February* 1958)

In the course of investigation of pain-inducing agents, Armstrong, Jepson, Keele & Stewart (1957) found that when blood plasma is exposed to a glass surface a pain-producing substance (PPS) is released. PPS, which is probably a polypeptide related to bradykinin (Rocha e Silva, Beraldo & Rosenfeld, 1949) and kallidin (Werle, Götze & Keppler, 1937; Werle, 1955), is also a powerful stimulant of smooth muscle. When plasma is left in contact with glass, PPS reaches a peak and then gradually disappears. When contact is interrupted the activity decays more rapidly. The substance is assayed by using its pain-producing effect on the exposed base of a blister or by its activity in stimulating plain muscle. For technical reasons the latter method has been used more extensively and has yielded results which are parallel with those obtained from pain experiments. The evidence presented in this paper is based mainly on the uterus-stimulating effect, although the crucial points have also been confirmed by pain experiments. Accordingly, it will be more appropriate to refer to the active substance as 'plasma kinin', which term has been recently proposed by Gaddum, Hilton, Keele, Lewis & Schachter (Lewis, 1958).

The term 'plasma kinin' is, therefore, used in the same sense as was 'PPS' in the paper by Armstrong *et al.* (1957). It will, however, be shown that as a result of contact with a glass surface there also appears in plasma a second active agent which stimulates both the smooth muscle and pain receptors independently of the formed plasma kinin.

It has been suggested that the reactions leading to the release of plasma kinin may be related to those involved in the initiation of clotting (Armstrong *et al.* 1957; Margolis, 1957*a*). It was shown that the latter can be attributed to a precursor, which is converted into an active substance by contact with glass and certain other surfaces (Margolis, 1956, 1957*a*). The glass-reacting system was found in the globulin fraction and the term 'contact factor' was provisionally used to describe the clot-accelerating agent. Similar findings were described by Shafrir & De Vries (1956) who used the term 'clot-promoting activity'. Subsequent work showed that at least two stages are involved in the activation of clotting by glass (Biggs, Sharp, Margolis, Hardisty, Stewart & Davidson, 1958) and that only the first of these is directly concerned with contact. Similarly Armstrong *et al.* (1957) suggested that there may be two stages in the formation of PPS; the contact with glass may activate a proteolytic enzyme and this in turn may liberate PPS from another component of the plasma.

The object of the present paper is to produce evidence for the existence of these two stages and to show that the product of the initial contact reaction is necessary both for the liberation of plasma kinin and for activation of clotting by contact with glass and related surfaces. To avoid difficulties in terminology it is proposed to restrict the term 'contact factor' to the product of this initial reaction. 'Contact factor' is not identical with the 'clot-promoting agent', but gives rise to the latter after reacting with another coagulation factor.

Some of the material presented here was the subject of preliminary communications (Margolis, 1957*b*, *c*).

METHODS

Unless otherwise stated all operations involving blood and its components were carried out at room temperature (approx. 20° C) using siliconed or 'Polythene' apparatus. Preparations which had not been exposed to glass surface will be referred to as 'intact'.

Human venous blood was mixed with 20% trisodium citrate (0·2 ml./10 ml. blood) and freed from cells by repeated centrifuging. Ammonium sulphate fractions were dialysed during the night at 4° C against citrated saline (3·8 g trisodium citrate, 8·5 g NaCl/l.)

Samples of plasma, etc., were activated by rotation with an equal weight of acid-washed glass microspherules ('ballotini') providing a surface area of 200 cm^2/g, and carefully removed, avoiding any transfer of ballotini with the plasma. In other experiments, where maximal activation was desired and the removal of the contact surface was not essential, instead of ballotini, powdered glass or light kaolin (hydrated aluminium silicate) was added to the plasma (10 mg/ml.) and the samples, including the powder, were tested on the uterus.

Clot-promoting activity was measured by adding standard volumes of the test plasma to intact plasma, recalcifying the mixtures with $CaCl_2$-platelet reagent and recording the coagulation time (see Margolis, 1957*a*). The correcting effect of various preparations of plasma was assessed by a modification of the 'kaolin clotting time' technique: 0·2 ml. amounts of the test plasma were mixed with 0·05 ml. of the added plasma preparations and 0·05 ml. of kaolin suspension (40 mg/ml.) After incubation for 2 min at 37° C, 0·1 ml. of a suitable dilution of lysed platelets and 0·1 ml. of 0·05 M-$CaCl_2$ were added in that order (see Margolis, 1958*a*).

Plasma kinin was assayed by applying the test substance (usually 0·1 ml.) to an isolated rat uterus suspended in a physiological salt solution in a 5 ml. organ bath set at 30° C. The length of the applications is indicated by the two marks under each tracing. The organ bath was washed out three times after each application while the drum was left running. The latent period of response was the main index of activity, the magnitude of the contraction, although usually related to the latent period, being of secondary importance for reasons discussed below. Application of glass-activated plasma to a freshly prepared uterus was often followed by a prolonged rhythmic activity. When necessary this was countered by shortening the period of application to 30 sec and/or by several preliminary applications of highly activated plasma. In later experiments it was found that a single preliminary application of soya bean trypsin inhibitor (SBTI) (0·5–1 mg/ml. bath fluid) for 1 min completely and irreversibly eliminated the rhythmic after-activity (see text).

For *pain-producing activity* the preparations were assayed on an exposed cantharidin blister base (for details of these bio-assays see Armstrong *et al.* 1957).

The indirect method for the assay of *contact factor* will be described in the text.

RESULTS

Two stages in activation of plasma kinin

When plasma was exposed to glass ballotini as described, maximum plasma kinin activity was reached in about 2 min. Thereafter the kinin decreased and it disappeared within 10–15 min. When a sample of this kinin-free plasma was removed and immediately added to intact plasma new kinin was released (Fig. 1). Evidently as a result of contact, something has appeared in the plasma, which can liberate kinin after an interaction with another plasma component.

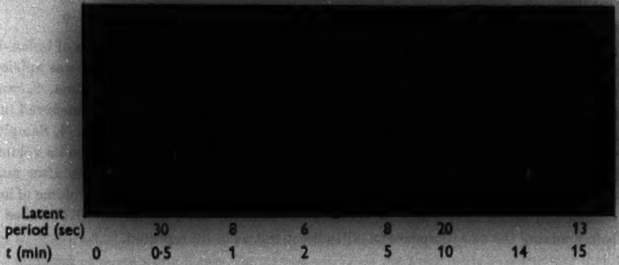

Fig. 1. Direct and indirect formation of plasma kinin. Rat uterus. 0·5 ml. amounts of plasma were rotated with equal weights of ballotini for increasing periods (*t*). Up to 14 min 0·1 ml. samples were applied directly to rat uterus in a 5 ml. organ bath. 0·1 ml. of the sample activated for 15 min was mixed with 0·2 ml. of intact plasma and 2 min later 0·1 ml. of this mixture was applied to the uterus (see text).

These findings are consistent with the hypothesis of Armstrong *et al.* (1957). The active substance which is still present after the kinin has disappeared from contacted plasma could be a proteolytic enzyme, and the component supplied in the intact plasma could be a specific substrate from which kinin is released. Since there is still no direct evidence regarding the nature of the reaction between these two, no assumptions will be made as to which is an enzyme and which the substrate. In general terms it can be stated that an inactive precursor ('component A') is converted by contact with glass into an active agent, which will be referred to as the 'contact factor'. The other substance(s) with which the contact factor reacts will be referred to as 'component B'.

To study the properties of contact factor without interference from formed plasma kinin, plasma must be first depleted of component B. This is done by a preliminary brief exposure to glass.

The minimal amount of glass contact, sufficient to deplete plasma of component B, was determined by repeated trials. B-depleted plasma could be prepared consistently by rotating plasma for 5 min with 1/7 of the usual quantity of ballotini (i.e. about 30 cm²/ml.) and leaving the decanted plasma in siliconed tubes for 2–4 hr at room temperature. Such plasma did not release any kinin when re-exposed to glass but could still be a satisfactory source of the contact factor. The stages of preparation of plasma depleted of component B are shown in Fig. 2. Records 5 and 6 show the development of kinin in a mixture of 1 part of glass-contacted, B-depleted plasma with 2 parts of intact plasma, and represent the second stage in the formation of kinin. It is obvious that this reaction is not instantaneous, as there was no detectable kinin activity in the mixture within 10 sec of bringing the two components together. By repeated estimations of the kinin level in such a mixture the activity was found to reach its peak at about 2 min and to decline rapidly after that time.

Fig. 2. Plasma depleted of component B as a source of contact factor. Responses of isolated rat uterus to 0·1 ml. doses. Soya bean trypsin inhibitor (1 mg/ml. of bath fluid) was applied to the uterus for 1 min before commencing the tests (see Methods). 1, Intact plasma. 2, Sample (1) treated for 5 min with ballotini (30 cm²/ml.). 3, Duplicate sample (2) transferred into a siliconed tube and left for 2 hr at room temperature (= B-depleted plasma). 4, Sample (3) treated for 2 min with ballotini (200 cm²/ml.). 5, Sample (4) mixed with twice its volume of intact plasma and tested after 10 sec. 6, Duplicate sample (5) tested 2 min after mixing (= standard procedure for indirect test). 7, Sample (3) mixed with twice its volume of intact plasma and tested after 2 min. 8, Indirect test as in (6) but 1 mg/ml. of SBTI added just before mixing the two components. 9, Indirect test as in (6) repeated.

It is known that soya bean trypsin inhibitor (SBTI) prevents the activation of plasma by contact (Armstrong *et al.* 1957), but does not affect the response to kinin once it has been formed. The tracing No. 8 in Fig. 2 shows that when SBTI was added in the second stage of the reaction no kinin was produced.

It appears, therefore, that SBTI either inactivates the contact factor or in some manner prevents its reaction with the other component(s) of the system.

The time course of the second stage can be followed in more detail by extracting successive samples with boiling saline. The reaction is thus immediately arrested, the formed kinin is recovered in a protein-free solution (Armstrong *et al.* 1957) and the extracts, being relatively stable, can be assayed at leisure. The time relations established by this method (Fig. 3) agree well with the results of direct tests on the reacting plasma mixtures.

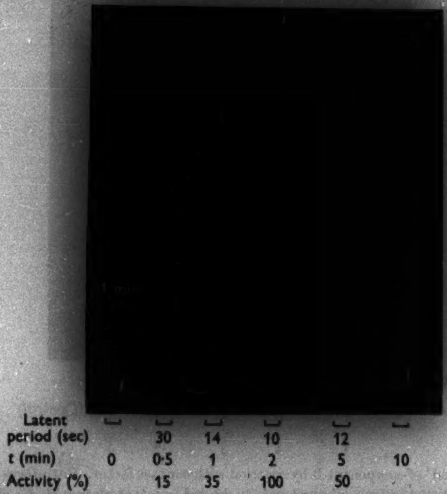

Fig. 3. Time relations of the second stage in the activation of plasma kinin. Rat uterus. Plasma depleted of component B (see Fig. 2) was activated for 2 min with ballotini and then mixed with twice its volume of intact plasma. 1 ml. samples of this mixture were removed at intervals (*t*) and immediately added to 2 ml. of boiling 0·9 % NaCl solution. After centrifuging to remove the coagulated protein, 0·2 ml. amounts of these extracts were tested on the uterus. The relative percentages of activity were calculated by reference to the responses to dilutions of the 2 min sample.

Formation of plasma kinin by the two-stage method was also demonstrated by testing the saline extracts of the reacting mixture on the pain area. Here also little activity was present in the initial sample as compared with the extract obtained after the two components were allowed to react for 2 min.

On the basis of these results the procedure used in the tracing No. 6 of Fig. 2 was adopted as the standard method for the indirect assay of the contact factor: one volume of the variously treated preparations was mixed with 2 vol. of intact plasma and the released kinin was assayed on the uterus after 2 min.

‘Consumption’ of component B

The latent amount of contact factor in plasma must be well in excess of that necessary to react with all the available component B, otherwise the two-stage experiments would not have been possible. This is shown more directly in Fig. 4. Varying amounts of glass-activated plasma were added to intact plasma. After 3 hr the mixtures were treated with ballotini and tested for kinin activity.

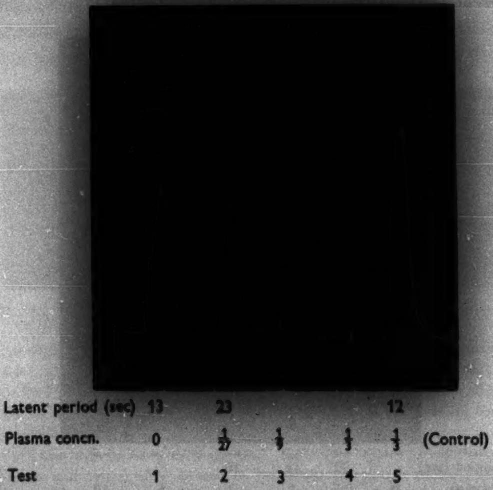

Fig. 4. ‘Consumption’ of component B by reaction with contact factor. Rat uterus. Plasma was activated with ballotini for 2 min and serially diluted in intact plasma (1–4); plasma concn., concentration of activated plasma in mixture. After 3 hr in siliconed containers the mixtures were again rotated for 2 min with ballotini, and 0·1 ml. amounts were tested on the uterus. A control sample (5) was also tested in which the activated and the intact plasmas were left for 3 hr in separate tubes and mixed immediately before the second activation.

The results indicate that the contact factor in a glass-treated sample can consume’ all the component B in about ten times its volume of intact plasma. In fact probably much less than 10% of the available contact factor is required, since the latter being itself unstable (see below) the amount present at any one time in glass-contacted samples represents only a fraction of the potential amount in the plasma.

Decay of contact factor

By subjecting plasma depleted of component B to various treatments some properties of contact factor were studied. When activated with the standard amount of ballotini (200 cm^2/ml.) the peak of contact factor activity was reached in 2–3 min. If at any stage the glass was removed the contact factor rapidly decayed (Fig. 5). A rough quantitative estimate of the rate of decay can be gained from a dose–response curve which showed that more than 90% of the activity was lost in 5 min at room temperature. At lower temperatures the decay is slower but even at 0° C it is not completely arrested. This instability of the contact factor (as well as that of kinin) must be taken into account in any attempt at a quantitative evaluation of the results; the actual yield of these substances must represent only a fraction of the potential yield.

Fig. 5. Decay of contact factor. Rat uterus. Component B-depleted plasma was activated for 2 min with ballotini and transferred into siliconed tubes. At various times, *t*, after removal from glass 1 vol. was added to 2 vol. of intact plasma. After a further 2 min 0·1 ml. of this mixture was tested on the uterus (indirect test).

Exhaustion of the precursor of contact factor (*component A*)

When contact with ballotini was continued, after 2–3 min the contact factor activity began to fall and was almost completely exhausted in 30–60 min (Fig. 6). The change was irreversible and contact factor could not be revived with fresh beads. Presumably all the available precursor of contact factor (component A) had been exhausted. This process is quite distinct from exhaustion of component B and requires more time to complete, as is implied in the results shown in Fig. 1.

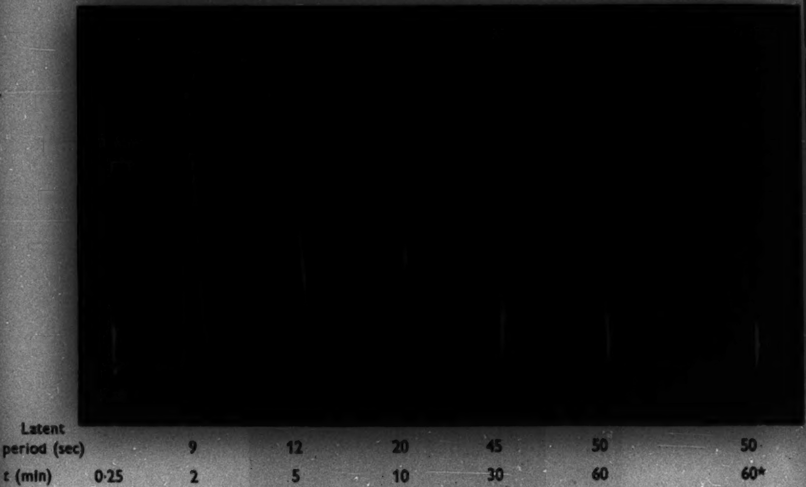

Fig. 6. Exhaustion of the precursor of contact factor. Rat uterus. 0·5 ml. amounts of B-depleted plasma were rotated with ballotini for various times, *t*, and tested on the uterus by the indirect method as in Fig. 5. * Plasma removed at 58 min and rotated for a further 2 min with fresh ballotini; indirect test as with the other samples.

Effect of heat and adsorbents

The formation of contact factor was not affected by pre-heating the plasma to 56–58° C for 20 min. In fact the activity was increased, as the decay was slower in heated than in unheated plasma. Contact factor could not be formed from plasma heated at or above 60° C for 15 min. It may be significant that this sudden change is associated with gross changes in the electrophoretic pattern of plasma heated to 60° C (Fig. 7). Treatment of plasma with 20 mg/ml. of $Al(OH)_3$ or $BaSO_4$ did not significantly influence either activation or decay. Treatment with 10–50 mg/ml. of kaolin removed the ability to form contact factor, but this is presumably only another example of 'exhaustion', since kaolin itself is even more potent than glass in activating the contact factor. A similar effect, and for the same reason, was obtained by using powdered glass or quartz.

Contact factor in protein fractions

Protein fractions were precipitated by $(NH_4)_2SO_4$ from intact plasma and dialysed throughout the night (see Methods). Under these conditions kinin was not detected in any combination of the fractions exposed to a glass surface and the formation of contact factor could therefore be studied without the need for preliminary depletion of component B. It is not clear which part of the procedure destroyed the ability to form plasma kinin, but since the original plasma, dialysed in parallel with the fractions, could still release kinin,

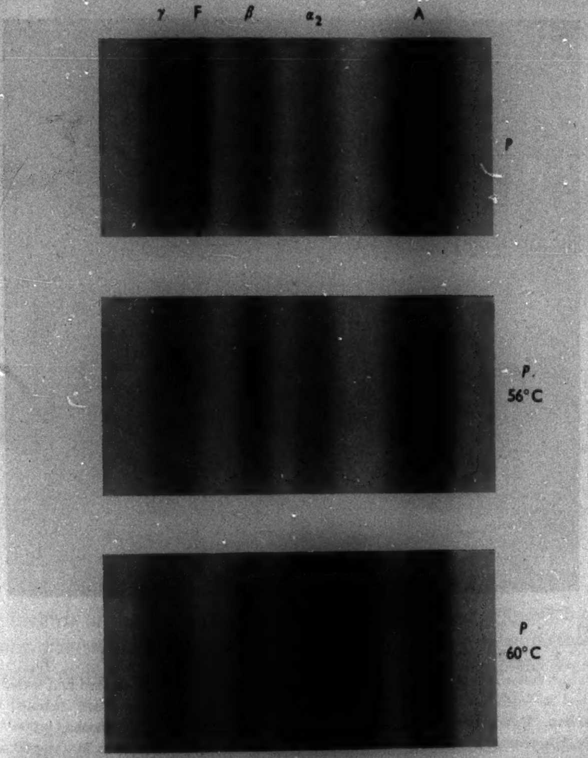

Fig. 7. Electrophoretic pattern of citrated plasma (*P*) heated for 20 min at different temperatures. Note disappearance of fibrinogen band (F) in sample heated at 56° C and the gross changes in the alpha globulin band at 60° C.

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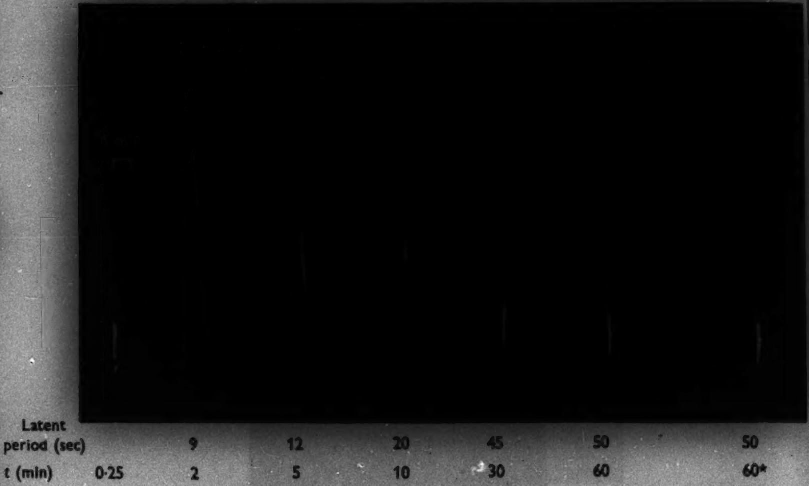

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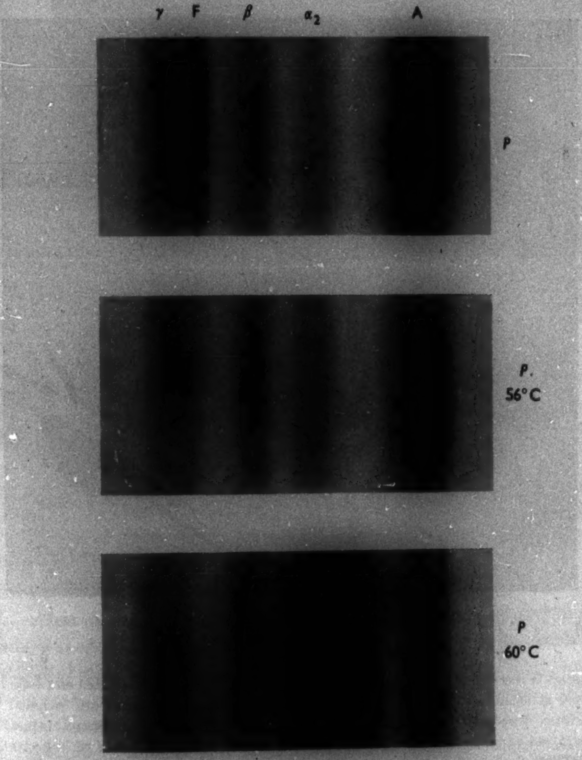

Fig. 7. Electrophoretic pattern of citrated plasma (*P*) heated for 20 min at different temperatures. Note disappearance of fibrinogen band (F) in sample heated at 56° C and the gross changes in the alpha globulin band at 60° C.

this change could not be the result of dialysis or ageing alone. The best separation was obtained by fractionation of plasma pre-heated to 56° C for 20 min. The fraction, precipitated between 25 and 45% saturation with $(NH_4)_2SO_4$, when exposed to glass developed contact factor activity comparable with that of whole plasma (Fig. 8). Relatively little activity was found in the remaining fractions. When unheated plasma was used as a starting point, the fractions precipitated below 25% saturation also contained considerable contact factor activity. As was shown in Fig. 5, contact factor rapidly decays in plasma. The decay is slower in pre-heated plasma. In the activated $(NH_4)_2SO_4$ fraction it was relatively stable (Fig. 9*b*) but when plasma was added to such fractions

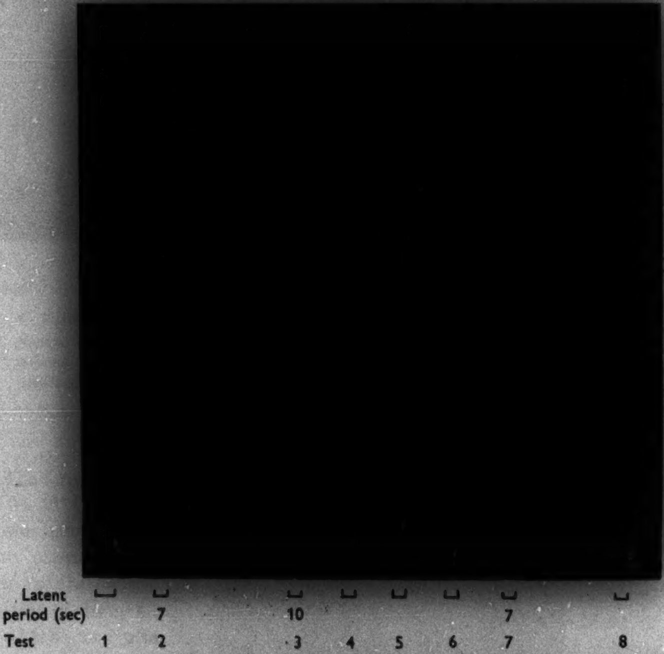

Fig. 8. Contact factor in a globulin fraction of plasma. All samples were activated and tested as described in legends to preceding figures. Fresh and dialysed plasmas are included for comparison. Direct tests: 0·1 ml. applied to the rat uterus. 1, Fresh plasma, intact. 2, Sample (1) activated by shaking with ballotini for 2 min. 3, Plasma heated at 56° C for 20 min and dialysed during the night, activated. 4, Sample (3) intact. 5, 25–45% saturated $(NH_4)_2SO_4$ fraction of (4), intact. 6, Sample (5) treated with ballotini for 2 min (note absence of response). Indirect tests: 7, Sample (6) added to intact plasma and tested after 2 min. 8, Sample (5) added to intact plasma and tested after 2 min.

the rate of decay again increased (Fig. 9*a*). These facts can be interpreted by postulating the presence in plasma of an inactivator which is relatively heat-labile and is not present in the same protein fractions as the activating system. The mechanism of inactivation of the contact factor is not the same as that of plasma kinin. Decay of kinin is inhibited by disodium edetate (EDTA) (Armstrong *et al.* 1955), which has no effect on the decay of the contact factor.

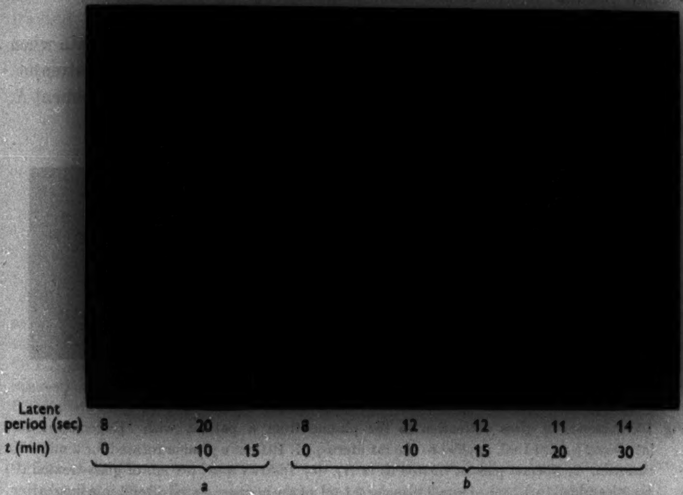

Fig. 9. Inactivation of contact factor. Rat uterus. (*a*) Equal volumes of the globulin fraction (cf. Fig. 8) and of B-depleted plasma were separately activated for 2 min with ballotini and then mixed; at intervals (*t*) samples of the mixtures were added to twice their volumes of intact plasma and 0·1 ml. amounts were applied to the uterus after 2 min incubation. (*b*) The samples were activated and tested as in (*a*) but the fraction and the B-depleted plasma were kept separately and mixed only immediately before each addition to the intact plasma.

Contact factor in pathological plasma

Because of the suspected relation with blood clotting, formation of plasma kinin was studied both by the direct and by the two-stage methods in the plasma of patients with various coagulation deficiencies. The reaction was found to be normal in cases of haemophilia, Christmas disease, deficiency of plasma thromboplastin antecedent (P.T.A.) as well as in factor VII deficiency and thrombocytopenia and in patients under phenindione treatment (J. W. Stewart, personal communication). Since Biggs *et al.* (1958) have shown that plasma from a case of 'Hageman trait' (cf. Ratnoff & Colopy, 1955) did not develop any clot-promoting activity after exposure to glass, it was of particular interest to test such plasma for the production of kinin. Fig. 10 shows that there was no release of plasma kinin when this plasma was treated with ballotini or kaolin. A two-stage analysis indicated that this was due to the inability to form contact factor and not to the lack of component B, and when a normal source of contact factor was added to the patient's intact plasma, kinin was formed in normal amounts. As a corollary to this and in contrast to normal plasma, it was found that 'Hageman' plasma was not depleted of component B by treatment with ballotini.

Further, glass-treated 'Hageman' plasma also failed to produce pain when tested on an exposed blister base (D. Armstrong, personal communication).

It is therefore reasonable to conclude that in 'Hageman trait' component A, that is, the precursor of the contact factor, is absent.

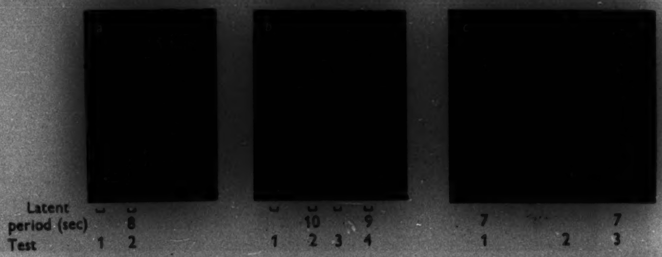

Fig. 10. Absence of glass activation in the plasma from a patient with 'Hageman trait'. (*a*) *Direct tests* (0·1 ml. applied to the rat uterus). 1, Patient's plasma rotated for 2 min with ballotini. 2, Normal plasma treated as in (1). (*b*) *Indirect tests*: 0·1 ml. of glass-treated (*G*) samples added to 0·2 ml. of intact plasma; 0·1 ml. of the mixture tested after 2 min incubation. 1, Patient's plasma (*G*) + normal, intact. 2, B-depleted, normal plasma (*G*) + patient's, intact. 3, B-depleted, normal (*G*); control, tested directly as in (*a*). 4, B-depleted, normal (*G*) + normal, intact. (*c*) Plasma activated with kaolin (10 mg/ml.) for 1 min, 0·1 ml. tested directly (see Methods). 1, Normal. 2, Patient's. 3, Normal. The tests under (*c*) were performed with a different sample from the same patient 7 months after those in (*a*) and (*b*). 1 mg/ml. of SBTI was applied to the uterus before commencing this experiment (see Methods).

Relation between the initiation of clotting and the formation of plasma kinin

The possibility that plasma kinin itself contributes to the clot-promoting activity of glass was tested by adding to plasma potent protein-free extracts of the kinin and recalcifying the mixtures under standard conditions (see Methods). The results were entirely negative, there being no difference between the clotting times with added kinin and with saline control. On the other hand, the properties of the contact factor with respect to plasma kinin formation resemble those described in connexion with blood coagulation (Margolis, 1957*a*).

What is more important is that 'Hageman' factor is an essential component for both sets of reactions.

It has been shown that after activation by the glass surface 'Hageman' factor interacts with P.T.A. to give rise to clot-promoting activity (Biggs *et al.* 1958). The sequence of these reactions is thus parallel to that described above in relation to components A and B. The analogy may be carried still further. Just as component B is 'consumed' by interaction with the contact factor leaving behind an excess of available component A, so P.T.A. is lost long before 'Hageman' factor, and as a result of a similar interaction. Table 1 shows the corrective effect of added plasma on the clotting time of plasma deficient in these respective factors. It will be seen that, after 10 min treatment with ballotini, normal plasma has lost most of its ability to correct P.T.A. deficiency, but little if any of its ability to correct the 'Hageman' defect. Moreover, when 'Hageman' plasma was treated in the same way, its corrective effect on P.T.A.-deficient plasma remained unchanged, which implies that 'consumption' of P.T.A. does not take place in the absence of 'Hageman' factor.

TABLE 1. Exhaustion of P.T.A. by treatment of plasma with glass

	Added plasma (0·05 ml.)							
	P.T.A.-deficient		'Hageman' trait			Normal		
Test plasma (0·2 ml.)	0′ *G*	5′ *G**	0′ *G*	5′ *G*	10′ *G*	0′ *G*	5′ *G*	10′ *G*
P.T.A.-deficient	205	—	140	141	143	132	163	184
P.T.A.-deficient (5′ *G*)*	—	300	166	165	165	135	193	228
'Hageman' trait	—	—	800	—	—	105	115	115
Normal	90	—	93	—	—	90	—	—

Kaolin clotting times in seconds (see Methods) of plasma mixtures. The samples marked 5′ *G* and 10′ *G* were rotated for 5 or 10 min with equal weight of glass ballotini, transferred into siliconed tubes and left for 2 hr at room temperature. The samples marked 0′ *G* were not exposed to glass. *P.T.A.-deficient sample was treated with glass to accentuate the deficiency. Note (1) rapid exhaustion of P.T.A. but not of 'Hageman' factor by contact with glass; (2) better correction of P.T.A. deficiency by glass-treated plasma from 'Hageman' trait than by glass-treated normal plasma.

Thus there is a qualitative correspondence between the reaction sequences of clotting and of kinin formation in both their first and second stages. In the latter, however, the two sets of reactions have already diverged and the components involved are no longer identical. Apart from the fact that kinin formation takes place normally in P.T.A. deficiency, this is also illustrated by the different time courses in the development of clot-promoting activity, on the one hand, and of plasma kinin on the other. As is shown in Fig. 11 'Hageman' plasma is not significantly activated by contact with glass. P.T.A.-deficient plasma responds normally within the first 1–1½ min, but then stops short, whereas the activity of normal plasma continues to increase for another 5–10 min. The initial acceleration of clotting time may thus be attributed mainly to the activation of 'Hageman' factor and it corresponds in time with the formation of contact factor (cf. Fig. 6). In the second phase, P.T.A. becomes the limiting factor and this part of the reaction is slower than the parallel stage in the formation of plasma kinin (cf. Fig. 1).

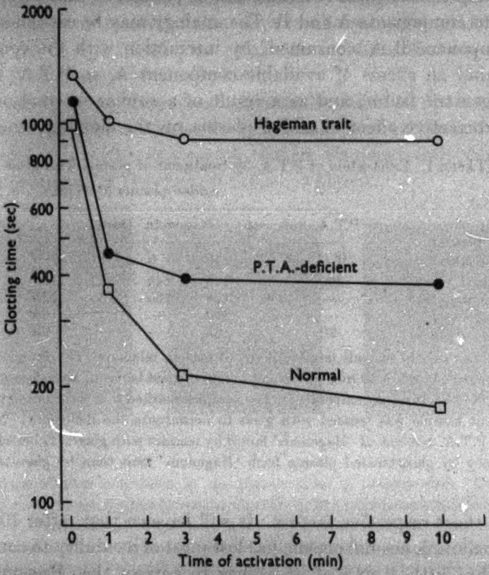

Fig. 11. Clot-promoting activity of 'Hageman trait', P.T.A. deficient and normal plasmas. 1 ml. of each plasma was rotated with ballotini. At intervals 0·1 ml. amounts were removed, mixed with 0·2 ml. of intact normal plasma and recalcified with 0·75 ml. of 0·1 M-$CaCl_2$ containing lysed platelets (see Methods). Ordinate, clotting time, log. scale.

Other effects of contact factor

'Direct' uterus-stimulating activity. In addition to prompt contraction due to plasma kinin, another, delayed type of response was also observed. This response merged with the kinin contraction when activated whole plasma was used, but it could be isolated by using B-depleted plasma.

The active principle differed from kinin in the following features: (1) It could not be recovered in protein-free extracts. (2) For a comparable latent period the contraction was much higher than with kinin and it was followed by 'spontaneous' rhythmic activity after washing. (3) The responses progressively decreased with repeated applications (Fig. 12). (4) They were irreversibly suppressed by a single application of SBTI to the uterus bath (Fig. 13).

Uterus preparations varied in their sensitivity to this type of stimulation. The effect occurred consistently with plasma activated by powdered glass, quartz or kaolin (cf. Fig. 12), which act in the same way as glass ballotini but are more potent because of the greater surface which they present to plasma. It was not always detectable with the usual doses (0·1 ml.) of ballotin activated plasma. When the effect was marked (as in Figs. 10*a*, 13), and when it interfered with two-stage tests for contact factor, the uterus could be desensitized with SBTI (see Methods).

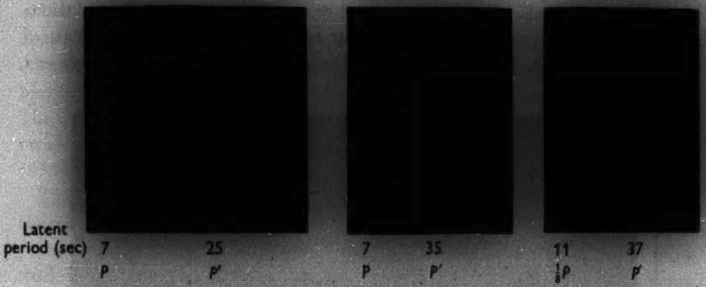

Fig. 12. 'Direct' response of the rat uterus to contact factor. Samples of plasma (*P*) and B-depleted plasma (*P'*) were activated for 1 min with 10 mg/ml. of kaolin. 0·1 ml. of each was alternately applied to the uterus for 20 sec and 60 sec respectively. *a*, Second application o *P* and *P'*. *b*, Fifth application. *c*, Sixth application: $\frac{1}{8}$ dose of *P* applied for 60 sec. Note the different patterns of response to *P* and *P'* and the decreasing effect of *P'* after repeated applications.

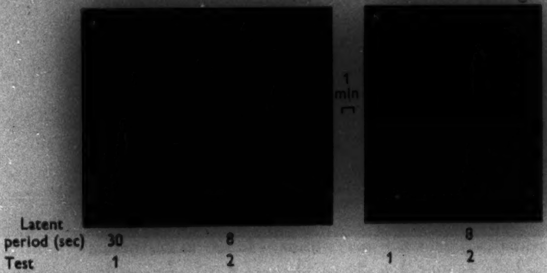

Fig. 13. Suppression by SBTI of the 'direct' uterus response. All tests samples were activated with 1 g/ml. of ballotini. Responses of rat uterus to 0·1 ml. doses. 1, B-depleted plasma; 2, whole plasma. Test (*a*), before SBTI; test (*b*), 20 min after a single application of SBTI (1 mg/ml. bath fluid) for 1 min. Note the composite nature of the contraction with whole plasma (2) and the suppression of the delayed phase by SBTI. In B-depleted plasma (1) the delayed phase was isolated and the whole of the response was suppressed by SBTI.

The delayed response could also be induced by activated globulin fraction containing contact factor, but not by plasma pre-heated to 60°C or by 'Hageman' plasma, neither of which can form contact factor. Hence, the activity appears to be dependent on the formation of contact factor.

This action of contact factor has been referred to as 'direct' by analogy to trypsin, which can stimulate the uterus either directly, or indirectly by releasing bradykinin from plasma globulins (Rocha e Silva *et al.* 1949).

Direct pain-producing activity. In agreement with the above findings, plasma depleted of component B and re-activated with glass induced pain in the blister base. Here also the activity was not recoverable in protein-free solutions and the effect was prevented by a previous application of SBTI to the base of the blister (Fig. 14). In these properties the active agent differed from plasma

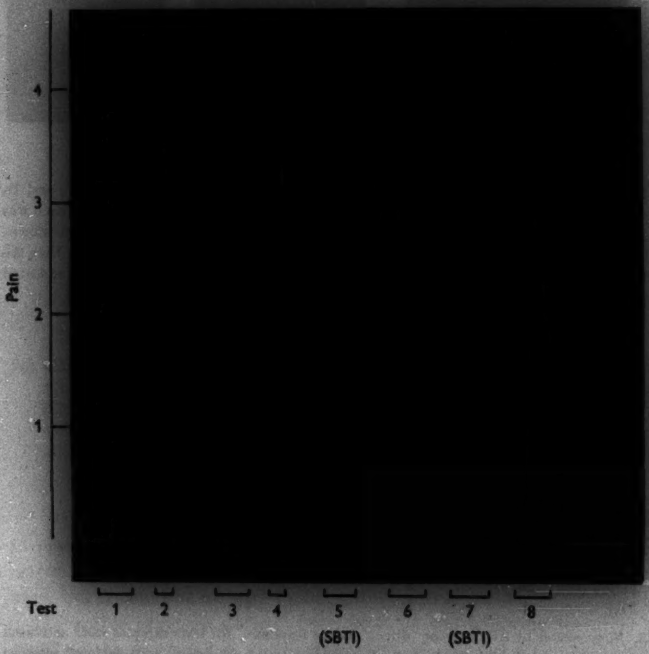

Fig. 14. 'Direct' pain response to contact factor and its inhibition by SBTI. 1, B-depleted plasma. 3, Intact plasma. 2 and 6, B-depleted plasma treated for 2 min with ballotini (=contact factor). 4 and 8, Plasma activated for 2 min with ballotini (=plasma kinin + contact factor). 5 and 7, SBTI (1 mg/ml.) applied to the area for 2 min and then washed off. Note the suppression by SBTI of response to contact factor (6) but not to kinin (8).

kinin. In pain experiments, as with the uterus, no response could be elicited with 'Hageman' plasma (D. Armstrong, personal communication). Thus it is evident that glass-activated plasma can stimulate both the uterus and the pain receptors in two different ways. The responses are normally superimposed but, under suitable conditions, they can be dissociated.

DISCUSSION

The investigation of the mechanism whereby kinin is released from plasma by contact with glass was guided by the results of a similar study on the initiation of blood coagulation (Margolis, 1957*a*). Most of the findings relating to clotting applied also to plasma kinin. Some apparent discrepancies led to a further investigation of the coagulation mechanism and helped to clarify the parts played by 'Hageman' factor and plasma thromboplastin antecedent (P.T.A.). The development in guinea-pig plasma of a factor increasing capillary permeability also appears to be related to the above phenomena (Margolis, 1958*b*).

There seems little doubt that glass contact initiates blood coagulation and the release of the plasma kinin via a common reaction. It is also likely that the permeability effect is an expression of the same initial change, although more work is needed to prove this, and in particular an investigation of this phenomenon in man is required. The essential change underlying these reactions is an alteration of a specific plasma component by contact with certain, as yet incompletely specified, types of foreign surface to form what has been termed the 'contact factor'.

The physical characteristics of active surfaces are being studied and the results will be reported separately. It is sufficient to say here that among the materials tested there is a close correlation between their clot-promoting and plasma kinin-releasing activity.

The first step of activation having been completed, contact factor in turn initiates diverging chains of reactions, the products of which are recognized by their different biological effects. A suggested sequence of events is outlined in Fig. 15. Unlike the subsequent stages of coagulation, activation of component A (i.e. 'Hageman' factor) to contact factor and the interaction of the latter with component B and with P.T.A. are not arrested by citrate or sodium edetate and are therefore independent of calcium ions.

Quantitative evaluation of the changes described is complicated by the fact that their products are rapidly destroyed by inactivators in plasma and the level of each is a resultant of a dynamic equilibrium between formation and decay. In the process of activation the precursor substances (i.e. components A and B and P.T.A.) are used up. The finding that component B and P.T.A. are exhausted long before the precursor of contact factor afforded a simple way of isolating the contact stage from the other stages of the reaction.

Component A, which has been identified with the 'Hageman' factor, is defined by some of its physical properties, by its function in relation to clotting and plasma kinin formation, and by its absence in a specific hereditary trait. P.T.A. is defined in a similar way except that all the evidence is based on its clot-promoting effect, since this factor does not participate in the formation of plasma kinin. There is as yet no known naturally occurring condition in which component B is missing; its definition is therefore limited to fewer criteria.

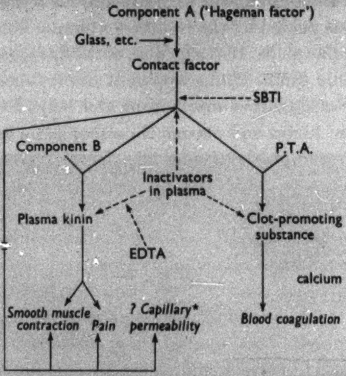
Component A ('Hageman factor')
Glass, etc.
Contact factor
SBTI
Component B
P.T.A.
Inactivators in plasma
Plasma kinin
Clot-promoting substance
EDTA
calcium
Smooth muscle contraction
Pain
? Capillary* permeability
Blood coagulation

Fig. 15. Relations between various biological effects of the plasma foreign surface reaction. Solid lines indicate activation, interrupted lines indicate inhibition. *Guinea-pig plasma (Margolis, 1958*b*).

It is recognized as a constituent of plasma which is necessary for the formation of kinin by contact factor and which is consumed in this reaction. The present data do not indicate the nature of the immediate substrate from which plasma kinin is split off, nor the role of component B in relation to this substrate. Thus component B could equally well be the immediate precursor of kinin or a co-factor in its production. For similar reasons it is not clear whether the 'direct' uterus and pain responses to preparations containing contact factor and free from kinin are to be regarded as due to kinin formation *in situ* or to some other action. Whatever the case may be, it should be emphasized that in plasma activated by glass there appear two distinct muscle-contracting and pain-producing substances: (1) plasma kinin, and (2) contact factor, which differs from the former in that it is antagonized by soya bean trypsin inhibitor and is not extractable in protein-free solutions.

An allied problem which still awaits investigation is the relation between the phenomena described in this paper and the activation of plasma by dilution, trypsin, saliva, urine and some snake venoms (Rocha e Silva *et al.* 1949; Tocantins, Carroll & Holburn, 1951; Miles & Wilhelm, 1955; Hilton & Lewis, 1955; Werle, 1955; Schachter, 1956; Holdstock, Mathias & Schachter, 1957; Stewart & Bliss, 1957). The observation that plasmin is a potent releaser of plasma kinin (Lewis, 1958) deserves special attention, since plasmin has also been shown to activate coagulation (Travis & Ferguson, 1951). It has also been shown that 'Hageman' factor is precipitated in the same protein fraction as plasmin (Ratnoff & Colopy, 1955). However, clot lysis in the presence of 'Streptokinase' has been found to be quite normal in 'Hageman' plasma (unpublished observations). 'Hageman' factor cannot therefore be identified with plasminogen or its 'proactivator'.

At present little is known about the physicochemical nature of contact activation, but the phenomenon seems to be specific enough to merit a functional definition. The proposed concept of the 'plasma foreign surface reaction' may be appropriate in that it directs attention to the possible physiological significance of these events. Thus it may be significant that there appears to be some correlation between the ability of a number of substances to activate contact factor in plasma and their activity in producing inflammatory tissue reactions *in vivo*. At one extreme there are such highly active surfaces as glass, quartz and kaolin which are known to provoke intense tissue reactions (Miller & Sayers, 1934; Kettle, 1935), at the other 'Polythene', $BaSO_4$, $Al(OH)_3$ and carbon which are relatively inert both in plasma and in tissues.

The most obvious objection against drawing such direct inferences is that the key component of the contact reaction is missing in persons with the 'Hageman trait' and that in spite of the deficiency the few cases of this trait described in literature (Ratnoff & Colopy, 1955; Frick & Hagen, 1956; Ramot, Singer, Heller & Zimmerman, 1956; Jim & Goldfein, 1957; Biggs *et al.* 1958) do not appear to have any haemostatic defects or other obvious physical disabilities. As has been remarked elsewhere (Margolis, 1957*a*; Biggs *et al.* 1958), haemostasis *in vivo* is not normally initiated by contact with glass or similar materials and an alternative mechanism must exist in the body. P.T.A. deficiency, however, is accompanied by a tendency to bleeding. We may therefore postulate that injured tissue releases substances which by-pass or substitute for contact factor and interact with P.T.A. to initiate normal coagulation in the body.

It is more difficult to evaluate the significance of the 'Hageman' defect in relation to plasma kinin and (possibly) permeability factor since the physiological and pathological role of these substances is still a matter for speculation. Plasma kinins, formed in different ways, produce vasodilatation and pain, which together with coagulation and increased permeability are amongst the cardinal features of inflammation (Menkin, 1956). How far all these changes depend on the reactions demonstrated in this paper cannot yet be stated but it may be said that cell-free plasma can be activated outside the body so that it could evoke most, if not all, of the features of inflammation.

Another aspect of the question is to what extent the phenomena detected in the plasma reflect what are essentially intracellular mechanisms. It is conceivable that the inflammatory responses *in vivo* are brought about chiefly by the changes in the cytoplasm of the cells but that, in the course of evolution, some of these mechanisms may have been transferred also to the body fluids (Robb-Smith, 1955; Biggs & Macfarlane, 1957). It is known, for instance, that cytoplasmic processes involving a sol-gel transformation, such as amoeboid movement and mitosis, are in many ways akin to blood coagulation (Monné, 1948; Heilbrunn, 1952). Thus it is possible that the plasma reactions, whilst of relatively subsidiary physiological importance, may serve as a model of intracellular reactions. If this view is correct, then the absence of the plasma contact-reaction in 'Hageman trait' could indicate an intracellular defect. It would be interesting to learn whether these patients show normal inflammatory responses to injurious agents, and especially to such materials as glass, quartz and kaolin. On the other hand, in view of the absence of clinical abnormality in this condition, it is probable that the defect is confined to plasma only. The precursor of contact factor or its equivalent might still be present within the cells so that cellular mechanisms of inflammation could occur normally. The 'Hageman trait' could then be considered to be merely an interesting genetic character, of no more importance to the affected individuals than is the inheritance of secretor or non-secretor state of the blood group substances.

Whilst the above speculations are still several steps removed from experimental facts, they suggest an approach to the study of inflammatory reactions to foreign surfaces and perhaps also to other forms of injury, and offer a working hypothesis as a guide for such an investigation.

SUMMARY

1. The mechanism of release of plasma kinin (originally termed 'pain-producing substance') by contact with glass was investigated. The reaction was resolved into two stages: in the first, a precursor in plasma (component A) is converted into an active agent, termed 'contact factor', in the second stage contact factor interacts with another substance (component B) to release plasma kinin.

2. The properties of contact factor were studied by an indirect method in which plasma was depleted of component B by brief exposure to glass. Contact factor was then allowed to react with the component B of intact plasma. The activity was assayed on the isolated rat uterus and also, for pain production, on an exposed blister base.

3. The formation of contact factor was not affected by prior treatment of plasma with $BaSO_4$ or $Al(OH)_3$ or by pre-heating at 56° C, but was prevented

by heating to 60° C for 15 min. Contact factor was also formed from a globulin fraction precipitated between 25 and 45% saturation with $(NH_4)_2SO_4$.

4. Contact factor is rapidly destroyed by an inactivator in plasma. The inactivator is largely removed by fractionation.

5. Prolonged treatment with large amounts of glass eventually exhausts plasma of component A.

6. Plasma from a case of 'Hageman trait' did not form contact factor when treated with glass or kaolin. Plasma from subjects of other coagulation deficiencies behaved normally in this respect.

7. Contact factor forms a connecting link between the initiation of clotting and the release of plasma kinin. Clot-promoting activity is produced by interaction of contact factor with plasma thromboplastin antecedent (P.T.A.). Plasma kinin is released by interaction of contact factor with component B, which is independent of P.T.A. (Fig. 15).

8. In addition to indirect excitation of plain muscle and pain receptors through the release of plasma kinin, contact factor acts independently on these receptors. Soya bean trypsin inhibitor suppresses this effect, but does not inhibit the action of plasma kinin.

9. Apart from glass, some other surfaces are potent activators of plasma. There may be a correlation between this action and the ability to provoke intense inflammatory reactions.

10. Plasma 'foreign surface reaction' may serve as a model for certain aspects of inflammatory reactions.

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Note added in proof. An intradermal test with 0·05 ml. of kaolin suspension (5 mg/ml.) was performed on a patient with Hageman trait. There was no trace of early whealing which in normal controls persisted for up to 2 hr. The onset of local erythema was delayed and the reaction less marked than in normal subjects. Thus Hageman factor may be a necessary component of an early 'humoral' phase of foreign surface response which may accelerate and intensify a later cellular reaction.

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MICRO-ELECTRODE STUDIES OF CEREBELLAR ELECTRICAL ACTIVITY IN THE FROG

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Adrian (1935) and Dow (1938) recorded spontaneous, low-voltage, high-frequency electrical activity from the surface of the mammalian cerebellum. The frequency of this activity was in the range of 150–250/sec. Their results have been confirmed and extended by Brookhart, Moruzzi & Snider (1950, 1951) with the use of micro-electrodes and subsurface recording. The latter group of investigators further identified spontaneous activity manifested by trains of high-voltage, uniform spikes in subsurface recording (Brookhart *et al.* 1950). They found that this spike activity could be abolished by induced ischaemia while the low-voltage, high-frequency waves continued unchanged (Brookhart *et al.* 1951). They concluded that the two types of activity arose from different mechanisms: the spike activity represented potential changes of an all-or-none character from single cerebellar neurones of the Purkinje and granule cell layers, and the low-voltage waves were fluctuations of membrane potentials not directly associated with conducted nerve impulses. Snider & Eldred (1949) found that this low-voltage wave activity was intrinsically maintained in the cerebellum, and was not due to reverberating circuits. They postulated that it was probably the result of spontaneous activity of Purkinje cells and/or the granule-basket cell combination.

The present investigation is a study of electrical activity of the cerebellum of the frog and the various mechanisms concerned in its genesis. Glass micro-electrodes, as described by Ling & Gerard (1949), were employed in the study.

METHODS

These experiments were performed on eighty decerebrated frogs (*Rana esculenta*) of a fairly uniform size. The decerebration was done by cutting through the head at the angle of the mouth. Occasionally, a small amount of tubocurarine was given intramuscularly to abolish movement. The cerebellum was exposed with bone rongeurs, and the connective tissue and meninges were cleared away with iridectomy scissors. The surface of the cerebellum was covered with liquid paraffin to prevent drying. The laboratory temperature was 17–20° C.

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The micro-electrodes were fashioned from glass tubing heated and drawn to a fine point and filled by boiling in 3 M-KCl (Nastuk & Hodgkin, 1950). The electrode tip was 0·5–1·0 μ in diameter and the electrode resistance was 10–20 MΩ. In some experiments ball-tipped Ag–AgCl electrodes were also employed. An oil-filled micromanipulator permitted exact placement and easy reduplication of any electrode position. The preamplifier employed (Matthews, 1953) allowed simultaneous recording of rapid and slow potential changes on the same tracing. In the recording of standing potentials within the substance of the cerebellum the micro-electrode was allowed to remain in each position until the reading had stabilized and any injury effects had disappeared. Further to check the accuracy of recording of standing potentials, the electrode was returned to the surface; if this reading varied by more than 2 mV from the original surface reading the results were discarded. Therefore, any variation in the earth or recording system was minimized. During the short interval required to obtain a series of standing potential readings, usually less than 3 min, there was very little variation of the standing potential at any one electrode placement. It was possible to leave an electrode in place for periods up to 10 min with only 1–2 mV drift, providing the recording equipment had been in operation for over 2 hr. The earth employed was the bared back muscles of the frog. Direct current stimulation, used to produce seizure-like electrical activity, was delivered from a dry cell through a variable resistance to two Ag-AgCl electrodes. The stimulating electrode tips were separated 3–4 mm and the recording micro-electrode was inserted between the tips.

Two schemes were used for direct current stimulation in studying changes in unit frequency with varying intensity and with varying polarity of current. To study the effects of changing current intensity, two Ag–AgCl electrodes were employed for stimulation; one was placed on the anterior surface of the cerebellum adjacent to the optic lobes and the other on the posterior surface at the rostral end of the fourth ventricle. The recording micro-electrode was placed in the cerebellum midway between the stimulating electrodes so that all three were then in a single parasagittal plane. To investigate the influence of the direction of current flow on unit frequency, the switch in Fig. 1 was employed with three stimulating electrodes. The circular trough was constructed of Perspex and filled with a solution made by adding a few crystals of copper sulphate to distilled water. The terminals leading to the three stimulating electrodes dipped into this solution at intervals of 120° around the circular trough. A triangular array of Ag–AgCl stimulating electrodes was used, with one on the anterior cerebellar surface and two on the posterior. The recording micro-electrode was inserted into the centre of this array. The terminals from the dry cell, situated at the ends of the rotating arm, also dipped into the trough. Thus, by a complete rotation of the arm, the axis of current flow rotated 360° around the point of recording at the micro-electrode tip. The intensity of the current varied only slightly at different positions of the rotating arm, but it was equal at every 60° interval when either end of the rotating arm was adjacent to one of the stimulating electrode terminals. It is only at these 60° intervals that readings were made, so that any error due to a slight drop in current intensity was avoided. The constantly changing polarity prevented any appreciable polarization at the stimulating electrodes.

Anatomical considerations

The cerebellum of the frog represents a transition between forms in which the lateral line system is the main equilibratory mechanism and those in which the vestibular apparatus serves that function. The lateral line system ceases to function after the larval stage, and the vestibular system with its cerebellar inflow remains in the adult frog. The main cerebellar efferent pathway arises in the cerebellar nuclei, which receives the Purkinje axons, and passes out through the brachium conjunctivum. The principal afferent supply is the spinocerebellar and vestibulo-cerebellar tracts. The histological features (Fig. 2) are similar to those of the mammal. There are well differentiated molecular, Purkinje, and granule cell layers. All the cellular elements are present as in the mammal with the exception of the basket, Golgi, and superficial stellate cells (Larsell, 1923; Glees, Pearson & Smith, 1958). The neuronal pattern of the frog's cerebellum also resembles the mammalian pattern of orientation, with the granule cell axons running transversely

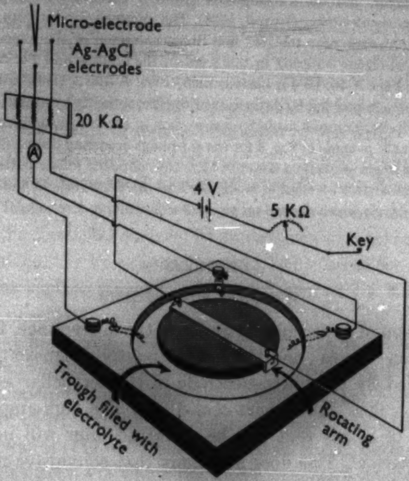
Micro-electrode
Ag-AgCl electrodes
20 KΩ
A
4 V
5 KΩ
Key
Trough filled with electrolyte
Rotating arm

Fig. 1. Diagram of switch employed to vary the direction of current flow.

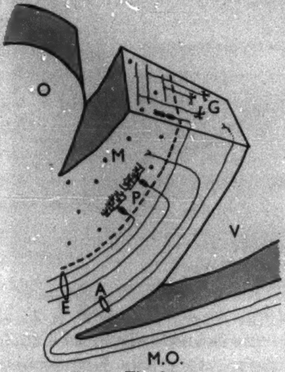

Fig. 2

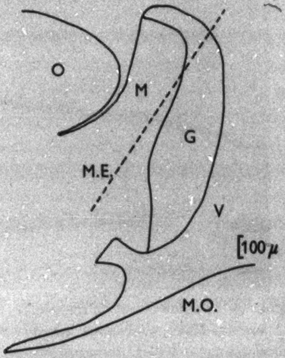
O
M
G
M.E.
V
100 μ
M.O.

Fig. 3

Fig. 2. Schematic diagram of combined sagittal and horizontal sections of the frog's cerebellum to show relationship of cellular elements. O, optic lobe; M, molecular cell layer; P, Purkinje cell layer; G, granule cell layer; E, efferent fibres; A, afferent fibres; M. O., medulla; V, fourth ventricle.

Fig. 3. Camera lucida drawing of parasagittal microscopic section of frog's cerebellum. O, optic lobe; M, molecular cell layer; G, granule cell layer; M.E., approximate direction of micro-electrode insertion; M.O., medulla; V, fourth ventricle.

in the molecular layer, and the Purkinje cell dendritic arborizations disposed in parasagittal planes within the molecular layer (Larsell, 1923). The micro-electrodes were introduced into the posterior cerebellar surface near the mid line from the region of the exposed fourth ventricle (Fig. 3). With the electrode entering in this fashion, the granule and Purkinje cell layers were traversed first (cf. Figs. 8–10, 15–17, Larsell, 1923; Figs. 1 and 2, Glees *et al.* 1958). Attempts at marking the electrode position by deposition of dyes from the tip were unsuccessful, but a rough estimation of the tip location was made by comparison of depth measurements with microscopic sections. No allowance is made in Fig. 3 for the shrinkage occurring during fixation of the microscopic sections. The frog's cerebellum does not have the confusing foliate pattern that exists in the mammal, hence histological correlation is simpler and more accurate in the frog. Furthermore, no conclusions are drawn which depend on precise localization in any cellular layer.

RESULTS

Wave and spike activity

In the description of experimental findings, the low-voltage, fast activity will be referred to as 'waves', and the brief, repetitive, high-amplitude potential changes recorded by the micro-electrode in the cerebellar substance will be designated as 'spikes' (Brookhart *et al.* 1951). The high-amplitude, high-frequency bursts of spikes that will be described later will be referred to as 'seizure-like activity'. This does not imply that convulsive movements were noted but is used merely as a designation.

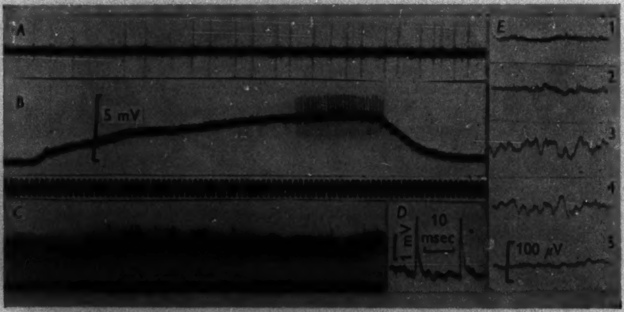

Fig. 4. *A–D*, micro-electrode recordings. *A*, spike activity at 420 µ, temp. 17° C; *B*, mechanoreceptor effect, calibration indicates d.c. change; time scale for *A* and *B*, 27 c/s. *C*, injury discharge at 300 µ; time scale, 50 c/s. *D*, spike action potential at 420 µ. *E*, bipolar Ag–AgCl surface recordings: (1) 15 min after ether anaesthesia; (2) 23 min after ether; (3) 28 min after ether; (4) 45 min after ether and 17 min after 0·2 mg intramuscular tubocurarine; (5) 3 min after surface application of 2 % cocaine; sweep time, 50 msec.

The micro-electrode recordings from the surface or from the substance of the cerebellum (Fig. 5, column 2) were similar to surface records taken between two Ag–AgCl electrodes (Fig. 4 *E*). The frequency of the waves was 150–250/sec and the amplitude 10–15 µV. The frequency and the amplitude of the surface waves were depressed during ether anaesthesia, were unaffected by intramuscular curare, and were promptly reduced by application of 2% solution of cocaine to the surface (Fig. 4*E*). Micro-electrode recordings from the cerebellar substance behaved in a similar manner, with the exception of slower disappearance of waves following application of cocaine to the surface. This is probably the result of delayed diffusion of the drug. Neither tactile stimulation of the extremities, stretching of muscle groups, nor faradic stimulation of mixed peripheral nerves affected the recordings of surface or depth activity. The wave activity was similar when recorded from any accessible portion of the cerebellar surface. If precautions were taken against drying or excessive damage, the wave activity could be observed for as long as 4 hr. The much slower surface waves in the frog described by Machne & Pupilli (1952) were not observed.

Spontaneous spike activity was recorded with the micro-electrode in the cerebellar substance (Fig. 4*A*). This varied in frequency from 8 to 40/sec, but was fairly constant from a particular focus. A rare finding was a unit with a frequency of 90–100/sec (Fig. 4*D*). Normally, the spikes were superimposed on the waves (Fig. 4*D*) and appeared in bursts lasting a few seconds with a brief silent interval in which the waves continued unaltered. There was no constant relationship between the phase of the wave activity and the appearance of spikes. It was much easier to find this spike activity in the superficial recording levels which corresponded to the granule and Purkinje cell layers. Again, peripheral nerve stimulation of various types did not alter the frequency or presence of cerebellar spikes. However, from a study of a single unit in a dense cell population such as this it would be impossible to say that some other units were not affected. Finding a unit responsive to peripheral nerve stimulation would be fortuitous both in the selection of the unit and in the stimulus provided. It was possible to record a single, active spike-producing unit for as long as 2 hr without any change in activity. Closer observation of one of these spikes (Fig. 4*D*), using long time constants, revealed that it was initially negative with a duration of 1–2 msec. The capacitance of the micro-electrode wall introduces some distortion in the spike contour (Brock, Coombs & Eccles, 1952), so that this does not represent a completely valid picture. The amplitude of the spikes varied, depending on the distance of the electrodes from the source, often increasing greatly in size from the point first encountered until the point of maximum amplitude was reached. Some of the smaller spikes recorded had a triphasic form which indicated conducted impulses that were probably from axonal sources.

The mechano-receptor property described in the ventral horn cell (Alanis & Matthews, 1952) was also encountered in the cerebellum (Fig. 4*B*). A spike could be detected 100 μ from the source and gradually increased in amplitude as the source was approached. Finally, when the electrode was advanced a few microns further, a prolonged negativity of the base line appeared and a rhythmic train of spikes occurred. The negativity would then reverse and the spikes would disappear. This manœuvre could be repeated several times without damage to the source and the spiking would reappear. Further advancement of the electrode resulted in a train of positive spikes with increasing frequency and decreasing amplitude (Fig. 4*C*), consistent with the characteristics of an injury discharge (Adrian & Matthews, 1934). Following this the spikes disappeared and were not detectable on re-positioning the micro-electrode at the point where they were originally most evident. Alanis & Matthews (1952) encountered the mechano-receptor in the spinal cord just before entering a zone in which there was a 60 mV negative deflexion of the base line, and then positive monophasic spike potentials were produced by reflex stimulation. These events were considered by Brock *et al.* (1952) to be indicative of intracellular recording. In this investigation frequent negative deflexions of the base line of 30–35 mV were encountered. However, these were only transitory, lasting less than a second, and the base line would rapidly return to the original level. This inability to record intracellular potentials for any length of time was probably the result of the small size of the cell bodies in the cerebellum as compared to the spinal motoneurones. There would be relatively greater damage to the membrane of the smaller cerebellar cell and more rapid displacement of intracellular potassium by diffusion from the micro-electrode tip (Nastuk & Hodgkin, 1950) than in the ventral horn cell. The surface membrane 'noise' (Brock *et al.* 1952) was occasionally encountered and could not be confused with the cerebellar waves by virtue of its much greater frequency and amplitude.

Wave activity and steady potentials

The wave activity in the decerebrate preparation was most prominent in the superficial layers. It decreased in amplitude and frequency upon insertion of the micro-electrode deeper into the cerebellar substance (Fig. 5, column 2). The application of 2% cocaine solution to the cerebellar surface abolished the wave activity at all depths (Fig. 5, column 3). The steady potentials with the wave activity superimposed could be measured by employing direct-coupled with capacitance-coupled amplification. The reference beam of the oscilloscope was adjusted to intersect the recording beam at the cerebellar surface and readings were taken at 200 μ intervals of depth (Fig. 5). In a total of twenty-three experiments in ten frogs, seventeen showed increased positivity of the steady potential with increasing depth as compared to the surface steady potential. This relationship was essentially linear, and the average reading at 1000 μ was 8 mV positive to the surface. In only two of the experiments were the depth readings consistently negative to the surface potential, usually 2–3 mV; and in four experiments the deepest recordings were positive to the surface but the superficial recordings at 200 and sometimes at 400 μ were 2–3 mV negative to the surface. The wave activity in all experiments was substantially less below the 600 μ depth. The record presented was typical of the majority of the experiments with the variation of slight relative negativity of the steady potential at 200 μ. After the application of 2% cocaine to the cerebellar surface in five frogs, the wave activity was abolished and the steady potential measurements were almost identical at all levels with the surface potential (Fig. 5, column 3), with an occasional variation of only 1–2 mV positive or negative to the surface.

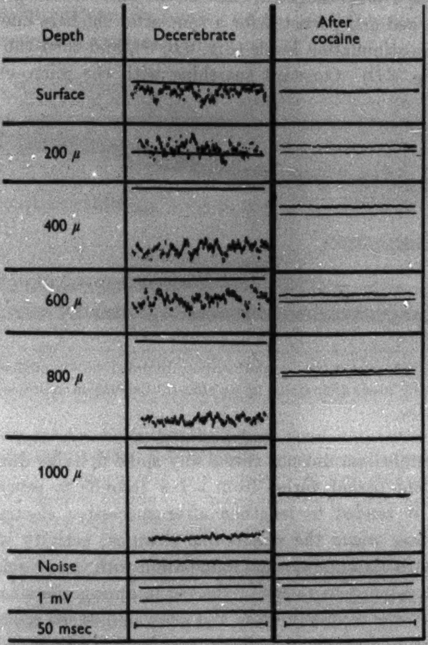

Fig. 5. Wave activity and steady potentials at different depths. Reference beam set to intersect recording beam at the surface. Third column following surface application of 2% cocaine. Downwards deflexion indicates positive d.c. shift. 1 mV calibration for d.c. change only.

Seizure-like activity

Electrical activity of a high-amplitude, high-frequency nature (Fig. 6) was observed during the course of these experiments. It corresponds closely to the electroencephalographic pattern observed in man and experimental animals during a convulsion. This occasionally appeared spontaneously after experimentation had been in progress for two or more hours. It was also observed following direct current stimulation of 10–100 μA delivered through two Ag-AgCl electrodes. It appeared as an increase in spike frequency concomitant with a 4–5 mV negative shift of the base line (Fig. 6*A*). A silent period then ensued and persisted for a time after the base line was returning and reached pre-stimulation levels (Fig. 6*B*, *C*), and then the spike activity reappeared (Fig. 6*D*). Constant searching with the micro-electrode in all layers of the cerebellum did not reveal any spike activity during this silent period. The silent period varied from a few seconds to several minutes in duration, and it tended to lengthen after successive electrical outbursts. In those instances where the seizure-like electrical activity was elicited by direct current stimulation, repeated stimulation with the original or increased current intensity failed to re-excite the tissue during the silent period.

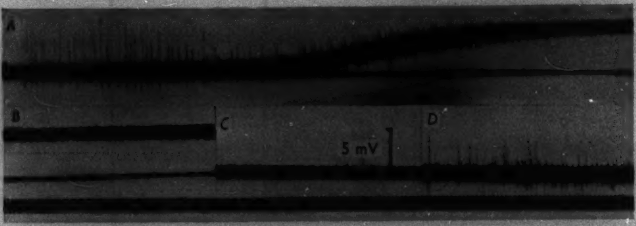

Fig. 6. *A*, spontaneous convulsive electrical activity recorded with a micro-electrode at 210 μ; *B*, 3 sec later; *C*, 16 sec after *B*; *D*, 12 sec after *C*. Calibration indicates d.c. change only Time scale 27 c/s.

Wave activity was recorded before and after an induced electrical outburst. This was superimposed on the direct current recording of the base line potential (Fig. 7). The wave activity did not alter appreciably during the period of spike extinction and slow positive drift of the base line. Unfortunately, it was impossible to record the waves during the stimulation and immediate induced seizure-like electrical activity. However, the widening of the base line (Fig. 6*A*) during a spontaneous electrical outburst may be increased wave activity, summed spike potentials or a combination of both.

The effect of direct current stimulation

Varying intensity of the current. Actively spiking units were sought with the micro-electrode, and only those units that gave spontaneous activity for two or more minutes were studied. Most of these units were initially negative and were encountered at less than 500 μ distance from the posterior cerebellar surface. The majority of these units also gave the mechano-receptor effect. From a study confined generally to these persistently firing units, it was likely that many of the spikes were of somatic origin because of their persistence without demonstrating injury effects, their relatively large size, and the possession of the mechano-receptor property. However, these criteria cannot be considered as strict evidence for somatic origin and it is possible that some of the units may represent axonal activity.

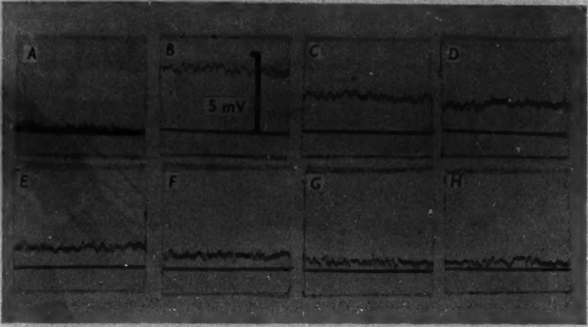

Fig. 7. Wave activity and d.c. changes following induced electrical convulsive activity recorded with a micro-electrode at 210 μ. Reference beam set to intersect recording beam at preconvulsive activity. *A*, spontaneous wave activity; *B*, 2 sec after application of 10 μA direct current to cerebellar surface; *C–H*, frames taken 1·6 sec apart. Calibration indicates d.c. change only; sweep time, 50 msec.

When one stimulating electrode was placed on the anterior surface and one on the posterior surface of the cerebellum, the current would flow approximately in the axis of the Purkinje and granule cell dendritic-axonal poles (Fig. 2). Analysing several units thus stimulated, three types of activity were observed. (1) The most commonly observed response was obtained with the anode placed anteriorly (Fig. 8 *A–F*). The frequency of the unit increased with increasing intensities of current, and spontaneous spiking from this unit could be silenced by reversing the current flow. Following the induced silence, the resumption of activity was either a return to normal spontaneous activity or a post-inhibitory rebound effect as demonstrated in the unit pictured (Fig. 8 *G*). (2) Less frequently a unit behaved as in (1), but with the current imposed in an opposite fashion, so that activation was accomplished with the cathode anterior and silence was induced with the anode anterior. (3) Rarely a unit was observed which did not alter with the current intensities employed.

It is difficult to correlate the current intensity and the unit frequency as other units are activated with greater current intensities. This can be observed in Fig. 8*E*, *F* in which different amplitudes of the spikes suggest that their source was from more distinct units or from units with a higher threshold of excitability. Other phenomena noted were the direct relationship of the train duration and the inverse relationship of the length of intertrain or non-spiking interval to the current intensity (Fig. 8*A–D*). This was a frequent but not consistent relationship encountered with current intensities just above threshold. In none of the experiments was there appreciable difference in latency of response with different intensities of current.

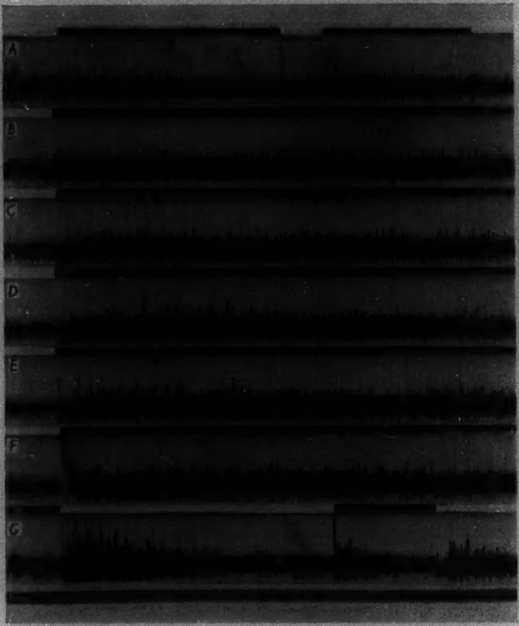

Fig. 8. Micro-electrode recording of spike activity at 500 μ modified by varying current intensity. *A–F*, anode anterior; successive current values, 18, 27, 36, 45, 54, 63 μA. *G*, same unit with anode posterior; current, 3 μA. Black line above record indicates stimulation. Time marker, 27 c/s.

Effect of varying current polarity. In order to ascertain the effect of current polarity on unit activity, the switch shown in Fig. 1 was employed. Single spontaneously active units were recorded with the micro-electrode. Slowly firing units were used so that slight changes in frequency could be more readily detected. The current axis was rotated through 360° using successively increasing intensities up to 90 μA until an alteration in frequency was obtained, and the point of maximum frequency response was noted visually on the oscilloscope and by means of the auditory monitor. The usual response (Fig. 9) was for the unit to be activated through several degrees of rotation, and for the point of maximum response to be midway between the point of activation and the point where the unit was no longer active. Usually, the initial frequency of response at the point of activation fell off rapidly, and then increased with further rotation of the switch to the point of maximum frequency of response. In four frogs, forty-four units of the type described in the previous section and satisfying some of the conditions cited above for possible somatic origin were tested. The units were confined to the first 500 μ from the surface in an effort to stay largely in Purkinje granule cell layer. Of these units, twenty-two gave a maximum response with the anode directly anterior, five with the anode directly posterior, four were unaffected with the intensities employed, and thirteen had a maximum response intermediate between the direct antero-posterior axis of the cerebellum. Of these thirteen, six gave a maximum response with the anode anterior to a transverse plane through the cerebellum and the remaining seven with the anode posterior to this plane. The effect could be obtained repeatedly on the same unit in every case.

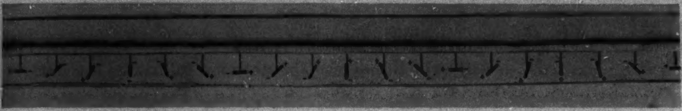

Fig. 9. Micro-electrode recording of spike activity at 480 μ modified by varying current polarity. + sign upward indicates anode on anterior surface. Current value, 25 μA; time, 0·6 sec between two successive positions.

DISCUSSION

The frequency, amplitude and response to drugs of the frog's cerebellar waves are strikingly like those demonstrated by Adrian (1935) and Dow (1938) in the cat, and are similar to the characteristic patterns of other mammalian cerebella described by Jacobs & Snider (1949). These similarities suggest that the electrical activity described is a characteristic of the cellular components and histological organization of the cerebellum, and is not closely related to the complexity of the nervous system as presumed from the phylogenetic scale. However, the spike activity found in the frog is of a much lower frequency than the 70–80/sec discharge recorded in the cat (Brookhart *et al.* 1950). This is possibly a reflexion of the lower body temperature of the poikilothermic frog. The effect of body temperature on the wave activity is not readily apparent, possibly because of the higher frequencies and the variable wave forms encountered in its analysis. The slower surface waves described in the frog's cerebellum by Machne & Pupilli (1952) are possibly analogous, as they suggested, to the slow waves encountered by Dow (1938) under abnormal conditions when the cerebellar surface was not protected.

The spike activity encountered in the cerebellum of the frog appears to be physiological as it is recordable for long periods of time and its characteristics differ from injury discharges. In addition, it probably arises from single cerebellar cortical neurones, and in some instances, from cell somata, because of the spike size and contour and presence in what is probably the densely cellular granule-Purkinje layers. In addition, the occurrence of the mechano-receptor phenomenon which was occasionally followed by negative base line shifts of a magnitude which could only be accounted for by intracellular recording of resting potentials, and the abolition of spike activity following the decline of the resting potential, also support this contention. Following somewhat different methods in their study on the cerebellum of the cat, Brookhart *et al.* (1951) also concluded that the spike activity was physiological and derived from single cerebellar neurones. Because of the technical difficulties in checking the placement of micro-electrodes, it is impossible to say what cell types are involved. However, it seems reasonable that only the Purkinje cells or large granule cells would give any resting potential, although only transitory values were obtained. Recently, Granit & Phillips (1956, 1957) in the cat, and Matthews, Phillips & Rushworth (1958) in the frog, have clearly analysed the response and wave form of the individual Purkinje cell. These workers also expressed difficulty in obtaining intracellular recordings, but did obtain large diphasic positive-negative discharges ('giant spikes') which they felt were from extracellular recordings of Purkinje cells. Although the amplitude of the spikes noted in the present study was not of the magnitude reported by Matthews *et al.* (1958) in the frog, it is noteworthy that the 'giant spikes' they obtained were similar in that: (1) deep anaesthesia obliterated them, (2) the discharge frequency under light anaesthesia was of the same order, and (3) the presence of the mechano-receptor effect noted above was also encountered by them.

The selective abolition of spike activity while wave activity continued following both spontaneous and induced seizure-like activity precludes the possibility that the waves are simply the product of summed spikes. This selective effect has been noted in the cat's cerebellum (Brookhart *et al.* 1951) and the cat's cerebral cortex (Li & Jasper, 1953). Another mechanism must then be invoked to explain the formation of waves. They may represent non-propagating membrane potential fluctuations as postulated by Brookhart *et al.* (1951) for the cat. Potential fluctuations not of the character of all-

or-none depolarization have been studied extensively (Barron & Matthews, 1938; Libet & Gerard, 1939, 1941; Renshaw, Forbes & Morison, 1940). To account for these, Gerard & Libet (1940) suggested the existence of a 'somatic potential' with each neurone polarized from the dendritic to the axonal pole; and, if the cells were oriented similarly, the whole sheet of cells would act like a nerve membrane and explain fluctuations and spread of electrical activity other than that of the usual conducted action potential. This hypothesis depends upon a potential difference existing between the dendritic and axonal poles and the existence of some neuronal orientation. These conditions appear in the frog's cerebral hemisphere (Libet & Gerard, 1941). A similar situation exists in the frog's cerebellum with the granule cell soma and dendrites concentrated in a zone which is several millivolts negative to the region in which its axons are situated. The disappearance of wave activity when these standing potential differences are abolished following cocainization suggests that a relationship exists between steady potentials and wave activity. However, there are objections, the principal one being the absence of a sudden change in the steady potential plotted through the cerebellum, which would be expected if any cell layers were acting as 'membranes'. It is possible that owing to the geometric complexity of the cell population and the variable plane of micro-electrode insertion, the 'membrane' was not penetrated at a right angle. The mechanism which maintains this potential difference between the soma and axon resulting in the 'somatic potential' is obscure, but the explanation of different rates of repolarization in various portions of a neurone following a conducted impulse has been offered to account for phenomena of this type (Burns, 1954). It then becomes difficult to relate persistent wave activity in the absence of spikes to the 'membrane' theory if the spikes or conducted impulses are necessary to maintain a somatic potential. Therefore, the observed facts do not support the hypotheses of summed spike potentials or cell aggregate 'membrane' as taking a part in the production of cerebellar wave activity in the frog. Adrian (1947) discussed several types of nervous activity which do not depend on the conditions mentioned and which may be concerned in the electrical pattern of the frog's cerebellum and possibly in the cerebella of higher forms. It is also possible that the D-responses and prepotentials demonstrated in the cerebellum by Granit & Phillips (1956) may in some way contribute to the final picture of this low-voltage wave activity.

The significance of the potential differences found between the superficial and deep layers of the cerebellum is not clear; however, it is possible that the resultant electrical field could exert a modifying influence on the interposed cells by maintaining their resting discharge, or by contributing a facilitatory or inhibitory effect on synaptic transmission in that region. Evidence for such a possibility is the graded effect of intensity and polarity of an applied current passing through the substance of the cerebellum. Brookhart & Blachly (1952) performed a similar experiment on the cat's cerebellum with the passage of a direct current along the dendritic-axonal axis of the Purkinje cells. They found that when the region of the dendrites was made positive to the axons the discharge frequency of the cells in that region increased. It is interesting to note that the majority of discharging elements in the frog's cerebellum acted in a similar fashion in regard to the polarity of the induced current flow.

The effect of altering current intensity on a discharging cerebellar cortical unit is much the same as that on cells elsewhere in the nervous system (Barron & Matthews, 1938; Burns, 1954). A peculiarity encountered was the variable duration of trains of spike discharges found with alterations in the small intensities of current passing through the frog's cerebellum. It may be that with low intensities of current the naturally occurring burst or interrupted activity is merely intensified by the addition of direct current, but that greater intensity of stimulation does not require additive effect of spontaneously occurring bursts and continuous activity is produced.

The abolition of spikes following induced or spontaneous seizure-like electrical activity as demonstrated here and in the cat's cerebellum by Mollica & Naquet (1953) was ascribed by them to 'exhaustion.' It appears to be closely related to the phenomena described by Dusser de Barenne & McCulloch (1939) in their study of convulsive activity and slow potential changes, as the slow potential drifts and concomitant spike activity recorded in the frog's cerebellum were similar to those obtained in the cerebral cortex of the cat. They found that increased neuronal activity and hyperexcitability occurred during the negative drift of the slow potential change and the reverse during the positive shift.

SUMMARY

1. The electrical activity of the frog's cerebellum was investigated utilizing subsurface micro-electrodes and surface Ag–AgCl electrodes.

2. Two types of activity were observed; a low-voltage, fast activity of 150–250/sec and a spike activity of 8–40/sec.

3. The spike activity arises from single cerebellar neurones.

4. Following 'seizure-like' electrical outbursts, spike activity is abolished and wave activity continues.

5. Steady potentials measured through the cerebellum reveal the granular layer to be negative to the molecular layer.

6. The occurrence and frequency of spike discharge are influenced by the intensity and polarity of direct current stimulation.

7. The 'seizure-like' electrical activity is accompanied by slow potential changes; negative drifts with facilitation and positive drifts with inhibition of spike discharges.

I wish to express my gratitude to Professor Sir Bryan Matthews for his interest and constructive criticism during the course of this work, and to Mr P. E. K. Donaldson for technical assistance.

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THE RELATION BETWEEN END-PLATE DEPOLARIZATION AND THE REPETITIVE RESPONSE ELICITED IN THE ISOLATED RAT PHRENIC NERVE–DIAPHRAGM PREPARATION BY DFP

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After the administration of di*iso*propylfluorophosphonate (DFP) to the isolated phrenic nerve–diaphragm preparation of the rat an increase is seen in the response to single indirect stimuli. This effect lasts only a short time and is not repeated after washing the preparation and addition of a second dose of DFP. The augmented contractions are due to repetitive responses of the muscle fibres (for references see van der Meer & Meeter, 1956). A similar effect after administration of eserine was studied by Eccles, Katz & Kuffler (1942). These workers concluded that the repetitive action was a catelectrotonic effect caused by the prolonged depolarization of the end-plate. They also found that the repetitive response eventually declined in size and duration, whereas the end-plate potential (e.p.p.) remained practically unchanged.

In the present investigation the relation between the twitch potentiation and the depolarization of the end-plate region was studied in the repetitively stimulated phrenic nerve–diaphragm preparation.

METHODS

The potential of the end-plate region

Strips of muscle were used, carefully dissected from the left hemidiaphragm of female albino rats weighing 120–150 g. The potential of the end-plate zone was measured as described by Fatt (1950). The strip of muscle was placed vertically in a bath. The surface of the liquid could be lowered at a constant rate. One non-polarizable electrode made contact with the upper end of the muscle (pars membranacea) the other electrode dipped into the liquid, the surface of which acted as a moving electrode. The potential along the muscle strip is recorded as the liquid is lowered.

The DC amplifier used had an input impedance of 10 MΩ (input to input) and was usually coupled to a pen recorder. In a few experiments the amplifier was also connected to an oscilloscope with an attachment for photography. These types of recording produced identical results.

Workers using this technique on intact muscles usually discard preparations showing more than

1 mV potential difference during the control observations. In the present investigations, however, the strips were dissected from a diaphragm. During the dissection slight injuries are inevitable and consequently preparations with potential differences up to 3 mV had to be included. Therefore some sort of control had to be obtained during the course of the experiment. The procedure adopted was to alternate each recording during stimulation with one after about 1½ min rest.

As a rule a trace was made every 5 min. The values of the potentials represented in the graphs are calculated by subtracting from the measured potential the mean value of the preceding and the following records taken during rest. It will be clear that the controls obtained during rest after the administration of DFP are not comparable to those recorded before adding this compound. Without DFP the effect of the spontaneous release of acetylcholine is certainly much less than after addition of DFP. The stimulus interval in all experiments was 2 sec.

In some of the experiments with pyridine-2-aldoxime-methiodide (PAM), the absolute reference point for the potential measurements was the potential found with the surface of the liquid at the pars membranacea of the muscle.

If the muscular contractions were to be recorded, a Starling heart lever (C. F. Palmer Ltd.) writing on a smoked drum was used.

Measurement of the 'blocking time'

After an e.p.p. evoked by an indirect stimulus, the impulse conduction in the muscle is blocked at the end-plate region for a certain period of time. The length of this period was measured in the same type of preparation as that described above. The muscle strip was mounted horizontally over four chlorided silver electrodes, two of which, situated close to the costal end of the strip, were used for stimulation; the two recording electrodes were usually placed at the other end. In a few experiments these electrodes were arranged on either side of the end-plate region. The nerve was stimulated through another pair of chlorided silver electrodes. The muscle was immersed in the bath solution and lifted above the surface when action potentials had to be recorded.

An experiment consisted of a series of tests with paired shocks at varying intervals, the first shock going to the nerve, whereas the second one of a pair stimulated the muscle directly. The action potentials of the muscle were photographed on the oscilloscope screen. By superimposing a series of sweeps (Fig. 7) it was possible to follow the development of the second muscle action potential (effected by the direct stimulus) with lengthening of the interval between the paired shocks. In Fig. 7*A* the muscle action potential following indirect stimulation is denoted by N, the potentials evoked by muscle stimulation by M. As a control, a trace of a directly elicited action potential not preceded by a nerve stimulus was also superimposed ($M_{max.}$). On the photographs the interval was measured between N and the M-spike attaining half the size of $M_{max.}$. Often the first action potential was immediately followed by a second smaller one, although one would expect all the muscle fibres to be blocked after the first action. However, during dissection a few fibres may have been deprived of their nerve connexion and consequently were not blocked after an indirect stimulus. In such a case the M-spike attaining half the maximal size was taken to be that one which reached the amplitude found as the mean of $M_{max.}$ and the lowest M-spike.

The nerve was stimulated during almost the whole experiment with supramaximal stimuli at 2 sec intervals. Muscle and nerve were both excited through RF output units (Schmitt, 1948) to isolate the stimulus from earth and to reduce stimulus artifact. The amplifier mentioned above was also used in these experiments, but to enable the superposition of successive sweeps a high pass filter was inserted into the circuit.

Solutions

The preparations were bathed in Krebs–Ringer solution prepared according to the method of Krebs & Henseleit (1932), kept at 37° and gassed with 95% O_2 and 5% CO_2. Di*iso*propylfluorophosphonate (DFP) and pyridine-2-aldoxime-methiodide (PAM) were supplied by the Chemical Laboratory of the National Defence Research Council T.N.O. Holland. Solutions were freshly made up every day. PAM was added as dry powder and quickly dissolved in the large volume of stirred bath fluid.

RESULTS

Potential of the end-plate region

In the first experiments the effect was studied of relatively high doses of DFP on the potential of the end-plate region. Fig. 1 represents the results of a typical experiment. To facilitate the interpretation, the recording during stimulation and the subsequent recording during rest have been given the same starting point on the recording paper. Fig. 1 reveals that soon after the administration of DFP (17 μg/ml.) the end-plate zone is depolarized during stimulation. Successive records show an increase of this depolarization followed by a decrease. About 100 min after the addition of DFP the depolarization has apparently disappeared. The first records taken during rest show no appreciable deviation from the controls. As time goes on the records taken during rest suggest a hyperpolarization of the end-plate region. If the atten-

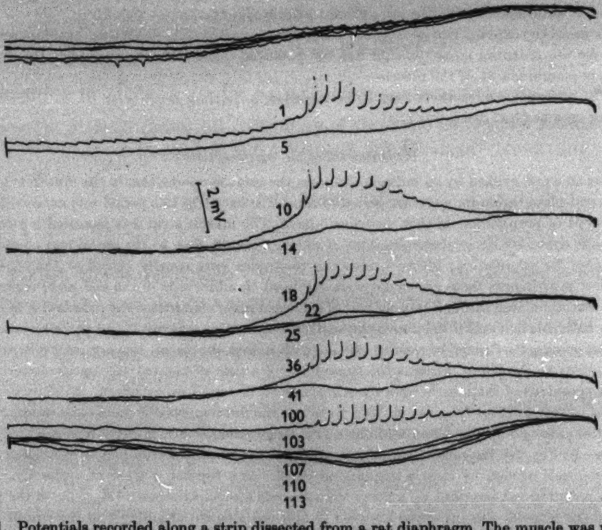

Fig. 1. Potentials recorded along a strip dissected from a rat diaphragm. The muscle was placed vertically in a bath containing Krebs–Ringer solution. The surface of the liquid acted as a travelling electrode when the fluid was allowed to run out, producing a record from the tendon end (right) to the rib end (left). After taking the upper bundle of control records, DFP was added to a final concentration of 17 μg/ml. The figures on the curves denote the time (min) after the addition of DFP. The curves showing end-plate potentials were taken during indirect stimulation (interval between stimuli 2 sec). Those without end-plate potentials were obtained after 1½ min rest.

tion is focused solely on the difference between the records obtained during rest and during stimulation, it can be concluded that the depolarization after addition of DFP soon reaches a maximum and then declines again.

With the high concentration of DFP used in the experiment of Fig. 1 the whole course of events is rather rapid. The administration of lower doses of this compound provides an opportunity to study more accurately the time relations between the development of the depolarization and the other phenomena. The time course of the twitch potentiation is illustrated in Fig. 2 for two DFP concentrations, 0·33 and 0·66 μg/ml., which are used throughout the remainder of this work. These records were obtained from preparations treated in the same way as those used for the measurement of the potentials.

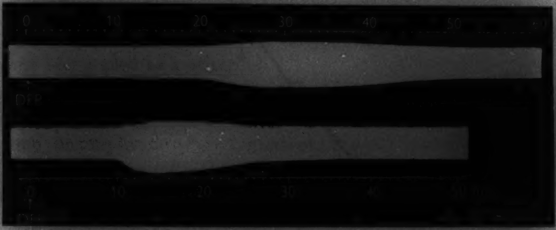

Fig. 2. Kymograph records of the contractions of the isolated rat diaphragm after administration of DFP. Upper curve, DFP concentration 0·33 μg/ml., lower curve 0·66 μg/ml. Indirect stimulation with 2 sec interval between stimuli.

Fig. 3 and the left-hand curve in Fig. 5 show the time course of the depolarization in the DFP concentrations just mentioned. It will be noticed that in both curves the maximal depolarization is found when the contractions in Fig. 2 have returned to normal. The decline of the depolarization when the maximum is passed cannot be reversed by washing. Identical results were obtained in many preparations.

It must be mentioned here that electrical recording still reveals after-discharges in the muscle when the contractions have returned to normal. During the subsequent 15 min (DFP concentration 0·33 μg/ml.) these after-discharges become less distinct and finally disappear. Earlier experiments (van der Meer & Meeter, 1956) suggested that the twitch potentiation was caused exclusively by the repetitive response of whole motor units, fasciculations (Masland & Wigton, 1940), and that the after-discharges of the individual muscle fibres do not add appreciably to the height of the contractions. It is therefore presumed that the after-discharges seen when the contractions are normal again are confined to the individual muscle fibres and have to be called fibrillations rather than fasciculations (Denny-Brown & Pennybacker, 1938).

If the muscles are washed during the phase of twitch potentiation, they maintain the augmented contractions for a long time (van der Meer & Meeter, 1956). A second dose of DFP then reduces the contractions to the normal height. Fig. 4 represents the results of depolarization measurements during such an experiment. The depolarization of the end-plate region reaches an intermediate level which is sustained until a new dose of DFP is added and the curve consequently passes a maximum.

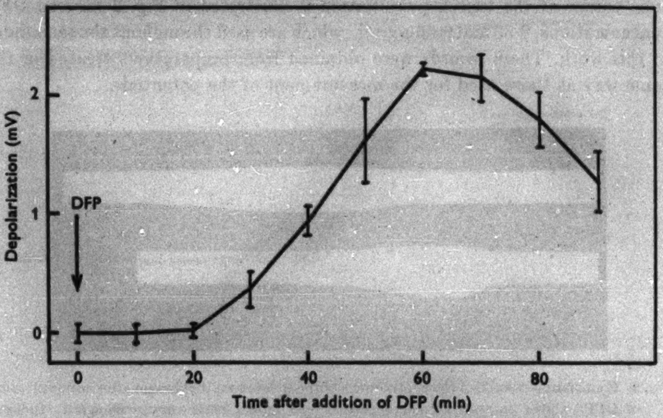

Fig. 3. Depolarization of the end-plate region of strips of rat diaphragm after addition of DFP to a concentration of 0·33 μg/ml. The results shown are averages of five experiments. The total length of the vertical bar denotes 2 × S.E. of mean. Continuous stimulation, interval between stimuli 2 sec. Depolarization measured as the difference between the traces taken during stimulation and during rest.

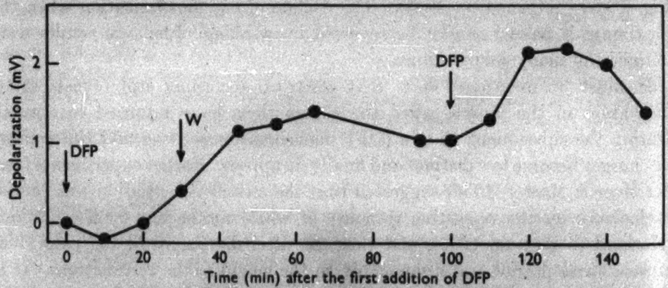

Fig. 4. Depolarization of the end-plate region of a strip of rat diaphragm as in Fig. 3. Final DFP concentration 0·33 μg/ml. The preparation was washed out at the crest of the twitch potentiation (W). A second identical dose of DFP was added 70 min after washing.

The amplitude of the e.p.p.

The general depolarization shown in Fig. 1 develops only if the nerve is stimulated, and it eventually decays. This depolarization might be related to a simple increase in amplitude of a single e.p.p., or related to prolongation of the e.p.p. The investigations described above enable some conclusions to be drawn about these possibilities. The amplitude of the summit of the e.p.p. cannot be measured in a contracting muscle because of the superimposed action potential of the muscle. It was found that the first reliable amplitude measurements could be made 9 msec after the onset of activity in the end-plate region. These measurements were carried out well on the declining part of the e.p.p. as in curarized muscles the summit of the e.p.p. is reached about 3 msec after the onset of activity. Before taking a record the surface of the liquid in the bath was carefully adjusted on the end-plate zone, the picture on the oscilloscope being used as a criterion.

The highest amplitude was always found at the time when the twitch potentiation was most pronounced. This result certainly does not imply that at that time the amplitude of the summit of the e.p.p. is also maximal. In fact, during the period of twitch potentiation many end-plates will, after the first excitation evoked by the nerve impulse, be activated a second time by an axon reflex, according to the mechanism described by Masland & Wigton (1940). If this happens, a second e.p.p. will be superimposed on the first, resulting in a higher amplitude found during the early decay. This phenomenon easily explains the high amplitude found during the moment of highest twitch potentiation. From that moment onwards the amplitude during the early decay decreases, whereas the over-all depolarization still increases. This can only mean that a progressive slowing of the decay of the e.p.p. is responsible for the gradual increase in the depolarization of the end-plate region.

In these investigations attention was also paid to the reversible depression of the e.p.p. described by Eccles & MacFarlane (1949). The results will not be described in detail, being only of minor interest in the scope of this paper. With a DFP concentration of 8 μg/ml. the depression was about 5%, a concentration of 33 μg/ml. reversibly depressed the (9 msec) amplitude 15–20% below normal. This indicates that even with the highest concentration used in the potential measurements (i.e. 17 μg/ml.) the reversible depression of the e.p.p. must have been very small.

The effect of pyridine-2-aldoxime-methiodide

The work of Wilson & Ginsburg (1955) has provided compounds which reactivate cholinesterase after inhibition by DFP. Pyridine-2-aldoxime-methiodide, PAM, was chosen and proved to be very effective in abolishing the depolarization, evoked by DFP, of the end-plate region of the rat diaphragm. The enlarged end-plate potentials were also lost and so the records obtained after 8 min PAM treatment followed by washing could not be distinguished from the controls taken before the addition of DFP. Fig. 5 shows that, after addition of PAM (final concentration 0·7 mM/l.) and subsequent washing, the whole sequence of events can be repeated by administration of a second dose of DFP. In all cases the second depolarization was lower than the first. The experiment represented in Fig. 5 (DFP concentration 0·66 μg/ml.) took 2½ hr. If a lower concentration was used (0·33 μg/ml.) the depolarization effected by the second dose was negligible in most cases. However, these experiments took 3–4 hr and a slow deterioration of the preparation may well have been responsible for the differences between the effect of the first and the second dose of DFP. It does not seem very likely that PAM is responsible for this deterioration. Holmes & Robins (1955) described a neuromuscular blocking effect of comparable concentrations of PAM but this effect could readily be reversed by washing the diaphragm.

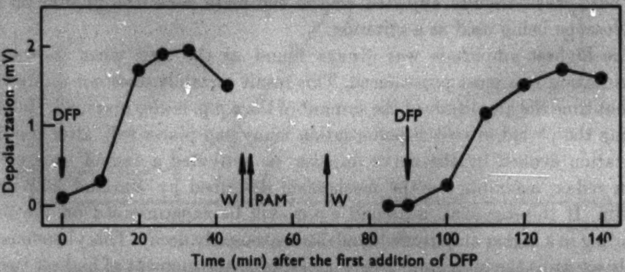

Fig. 5. Depolarization of the end-plate region of a strip of rat diaphragm after a dose of DFP (0·66 μg/ml.) twice that given in the experiments of Figs. 3 and 4. After washing (W) and treatment with PAM, DFP produces a similar result a second time.

The use of PAM provided a means of taking a control after the completion of an experiment. In this way the existence of a hyperpolarization, as suggested in Fig. 1, could be confirmed. Fig. 6 represents an experiment in which a marked hyperpolarization occurred and disappeared after PAM treatment. A comparable hyperpolarization was observed in three out of four cases. In these experiments remarkably little potential difference was found between the recordings taken before the administration of DFP and those after treatment with PAM, indicating a high stability of the preparations.

The conduction block at the end-plate

Kuffler (1942) has shown that the depolarization of the end-plate evoked by a nerve impulse prolongs the refractory period at the end-plate zone. The duration of the refractory period in this region might have a significant effect on the repetitive response elicited by DFP. However, estimation of the refractory period in the end-plate zone of a muscle is complicated by the fact that direct stimulation of this region is likely to activate nerve fibres. This difficulty could be overcome by the determination of the time during which conduction along the end-plate region is blocked (blocking time). The muscle was stimulated at one end and the recording electrodes were placed on the other end. Such a technique was also used by Burns & Paton (1951) to demonstrate the conduction block at the end-plate region of the gracilis of the cat, resulting from a depolarization by decamethonium or acetylcholine. In those experiments the depolarization obviously reached such a high value that in many muscle fibres a continuous conduction block was obtained that lasted several minutes. However, in the experiments described here the muscle always responded to nerve stimulation and only a short period of conduction block after the arrival of each nerve impulse at the end-plates was to be expected.

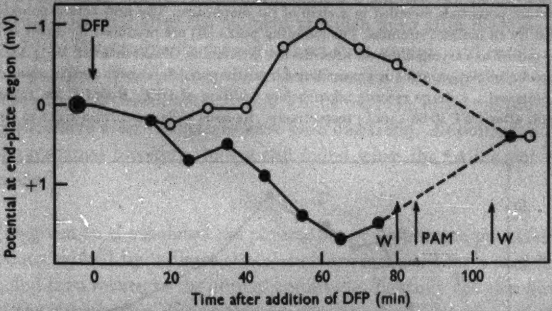

Fig. 6. Potentials found at the end-plate zone of a strip of rat diaphragm after DFP (0·33 μg/ml.). The open circles were obtained during stimulation, the filled after 1½ min rest. The last control was taken after washing (W) and treatment with PAM.

Therefore the time interval was measured between the action potential elicited by a nerve volley (N in Fig. 7) and the subsequent directly evoked action potential (M) attaining 50% of its maximal amplitude ($M_{max.}$).

By varying the time interval between the nerve and the muscle stimuli and by superimposing a group of oscilloscope sweeps, results like those represented in Fig. 7 are obtained. Fig. 7 *A* shows a control taken before addition of DFP. Fig 7 *B* was obtained 30 min after adding DFP (0·66 μg/ml.) and clearly shows a prolonged blocking time. Fig. 7 *C* represents the situation 130 min after the administration of DFP; the blocking time is shorter again notwithstanding notable deterioration of the muscle. The action potentials marked '$M_{max.}$' are obtained after a direct stimulus not preceded by a nerve impulse, and served as controls.

Fig. 8 illustrates the changes in the blocking time after addition of the two DFP concentrations in typical experiments. The maximum blocking time is found at the same time after administration of DFP as the maximum depolarization in the earlier experiments (Figs. 3, 5). In the experiments the results of which are shown in Fig. 8*A* the recording electrodes were situated on either side of the end-plate zone. Measurements carried out on the action potentials originating from the first electrode, placed between the stimulating electrodes

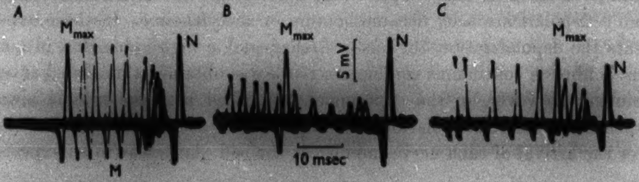

Fig. 7. Action potentials recorded in a strip of rat diaphragm. The first action potential (N) is evoked by an indirect stimulus. The following peaks (M) are produced by direct stimulation of the muscle at varying intervals after the nerve stimulus. Peaks denoted $M_{max.}$ are obtained by direct stimulation without a preceding nerve stimulus. Successive oscilloscope sweeps are superimposed. *A* shows records taken before addition of DFP, *B* and *C* are taken 30 and 130 min after DFP (0·66 μg/ml.) respectively. Records to be read from right to left.

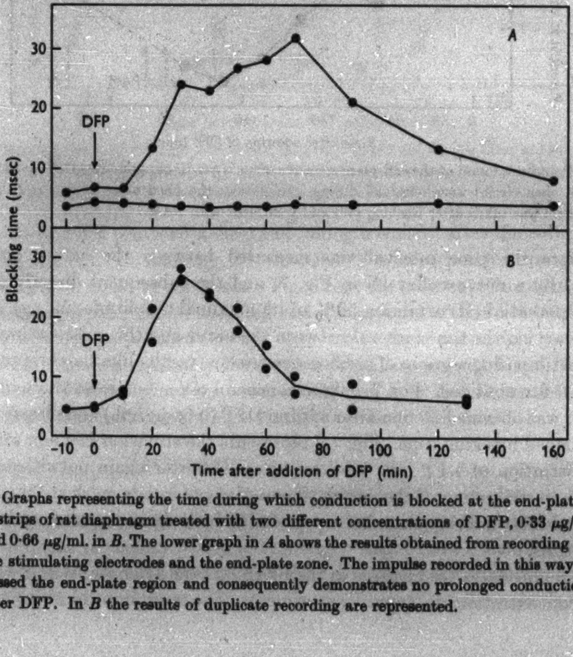

Fig. 8. Graphs representing the time during which conduction is blocked at the end-plate region of strips of rat diaphragm treated with two different concentrations of DFP, 0·33 μg/ml. in *A* and 0·66 μg/ml. in *B*. The lower graph in *A* shows the results obtained from recording between the stimulating electrodes and the end-plate zone. The impulse recorded in this way has not passed the end-plate region and consequently demonstrates no prolonged conduction block after DFP. In *B* the results of duplicate recording are represented.

and the end-plate, reveal an unchanged blocking time during the whole course of the experiment (lower curve). The action potentials picked up by the second recording electrode beyond the end-plate show the described lengthening of the blocking time (upper curve). This result proves that the part of the muscle lying between the two recording electrodes, i.e. the end-plate region, is responsible for the observed effect.

During the phase of twitch potentiation the action of a muscle fibre is often succeeded by one or more after-discharges. Such an after-discharge is again followed by a refractory period. If the direct stimulus arrives during such a second refractory period, the fibre will not respond. This absence of response, not being caused by the refractory period following the first action of the muscle, seriously complicates the measurement of the blocking time. On the photographs this phenomenon produces a sudden fall of the amplitude of the action potential after the moment at which most after-discharges occur (see Fig. 7*B*). Measurements carried out on such records will result in exaggerated values for the blocking time, therefore the blocking times measured during the phase of twitch potentiation will be too long, although preparations showing excessive after-discharges have been discarded. Notwithstanding this effect the maximal blocking time is still found when the twitch potentiation has vanished.

DISCUSSION

If DFP is given to the isolated rat phrenic nerve–diaphragm preparation, the contractions evoked by indirect stimulation are increased for a certain period of time, and then return to the normal amplitude. To study the changes taking place in the preparation during this process, the depolarization of the end-plate region was measured, as well as the period of time (blocking time) during which, after an indirect stimulus, conduction is blocked in this part of the muscle. The time course of these phenomena and the probable course of the cholinesterase inactivation (van der Meer & Meeter, 1956) in the presence of a DFP concentration of 0·33 μg/ml. is schematically drawn in Fig. 9.

The picture obtained may be explained in the following way. With increasing enzyme inactivation the amplitude of the e.p.p. is enlarged progressively and the rate of the decay is slowed. Consequently every e.p.p. will become superimposed on the tail of its predecessor, resulting in a steady depolarization. The augmented depolarization of the end-plates will lengthen the refractory period of the adjacent parts of the muscle fibres (Kuffler, 1942). This corresponds with an increased blocking time in the present experiments. If the decay of the e.p.p.'s is sufficiently slowed, after-discharges in the muscle fibres (fibrillation) will be evoked and retrograde transmission of this activity through the depolarized end-plates will lead to repetitive action of whole motor units (Eccles *et al.* 1942). The latter effect is responsible for the increase in height of the contractions (van der Meer & Meeter, 1956).

However, the blocking time increases gradually, presumably reflecting an enlargement of the area blocked after the arrival of a nerve volley. In the end the blocked area will be increased to such an extent that the after-discharges are prevented from generating an axon reflex through the end-plate. Consequently, the twitch potentiation will die out. This may also be partly caused by the decrease in number of muscle fibre after-discharges, although it was found that these are still present when the contractions have returned to normal. The gradual diminution of the repetitive activity of the muscle fibres presumably prevents the reappearance of augmented contractions when later on the blocking time shortens again.

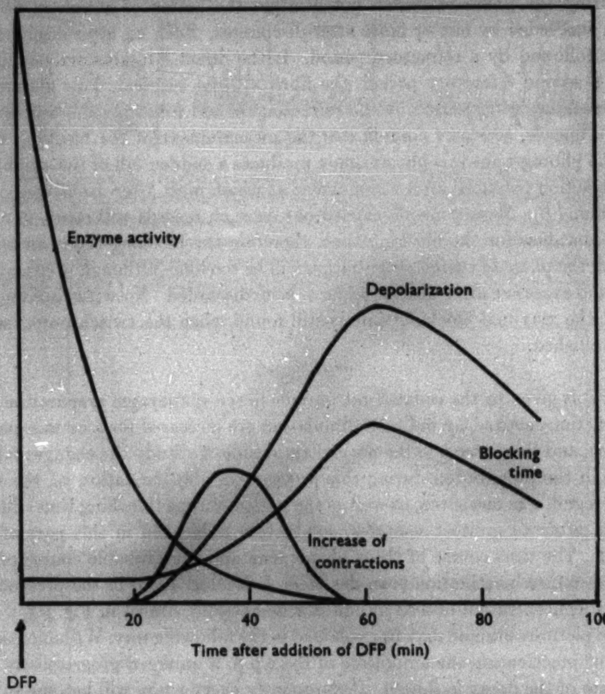

Fig. 9. Schematic representation of the time courses of the observed phenomena, for a DFP concentration of 0·33 μg/ml. The line showing the probable course of the cholinesterase inactivation is deduced from the results of earlier experiments (see text).

The remarkable fact that after-discharges occur in muscle fibres in which the end-plate is surrounded by a blocked area may be an analogy of the phenomenon described by Burns, Frank & Salmoiraghi (1955) in a muscle of which a part was treated with veratrine. The repetitive response in such a muscle was found to originate from the boundary between the normal and the veratrinized part. These authors suggest that the after-discharges are not evoked by a depolarization as such but by a difference in the rate of repolarization. A similar situation may well exist in the DFP-treated diaphragm, in which the after-discharges in the fibres may originate from the border of the depolarized (blocked) area. The progressive reduction in the number of these after-discharges cannot be explained at present. It is unlikely that the blocking time is an important factor in this process, as shortening of this time starts before the repetitive activity of the muscle fibres has disappeared.

The hyperpolarization observed in the preparations after a short time of rest resembles the phenomenon described by Burns & Paton (1951) and Douglas & Paton (1954); a previously depolarized end-plate region in the cat's gracilis repolarized after treatment with tubocurarine to such an extent that it became positive with respect to the adjacent regions. It might also be an analogy of the post-tetanic hyperpolarization observed by Ritchie & Straub (1957) in 'non-medullated' rabbit nerve fibres. There the collapse of a depolarization of relatively long standing resulted in a hyperpolarization of the nerve membrane. However, the methods used in the experiments described in this paper do not allow an accurate determination of the magnitude and the time course of the hyperpolarization and further work will be needed to reach a definite conclusion.

When the enzyme activity is reduced to a very low value the depolarization and the blocking time reach a maximum. The subsequent diminution of the amount of depolarization cannot be caused by a deterioration of the muscle, as in that case it would have been impossible to repeat the sequence of events after treatment with PAM. A spontaneous dissociation of the enzyme-DFP complex cannot be responsible, as the time course of this process (Hobbiger, 1951; Burgen & Hobbiger, 1951) is much too slow. It is unlikely that a depression of the e.p.p. by DFP is of much importance for the problem concerned, as this is only seen after higher doses and would have been reversed by washing. A possible explanation of the diminishing depolarization might be provided by the accommodation of the end-plate to acetylcholine by the presence of this compound in high concentration, a mechanism first suggested by Fatt (1950) and Douglas & Paton (1954) and studied extensively bv Thesleff (1955*a*, *b*) and Katz & Thesleff (1957).

SUMMARY

1. The effect of DFP on the depolarization of the end-plate region of the indirectly stimulated rat diaphragm has been studied.

2. It was found that the depolarization at first increases and subsequently decreases again to a comparatively low value.

3. Comparison of the time course of this effect with that of the twitch potentiation evoked by DFP revealed that the maximal depolarization coincides with the return to normal of the amplitude of the contractions.

4. The length of the period during which conduction of impulses in the muscle is blocked after the arrival of a nerve volley was found to increase with increasing depolarization and *vice versa*.

5. In many preparations a hyperpolarization was observed at the end-plate region after a period of rest.

6. The significance of these effects with regard to the after discharges in the muscle and the nerve after administration of DFP is discussed.

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VASCULAR AND METABOLIC RESPONSES OF THE LIVER TO INSULIN

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By means of the bromsulphalein method it has been shown in man that insulin hypoglycaemia is accompanied by an increase in liver blood flow (Bearn, Billing & Sherlock, 1952). These experiments, however, threw little direct light on the relative importance of hepatic artery and portal vein, nor could they really be regarded as conclusive in regard to the importance of nervous or endocrine factors.

The first object of the present work was, therefore, to apply the method of 'internal calorimetry', a heated thermocouple technique which depends on the measurement of thermal conductivity (Grayson, 1952; Hensel, Ruef & Golenhofen, 1954), to the fuller investigation of this phenomenon in rats and baboons.

The present work has had, too, a subsidiary object, namely, to determine changes in hepatic heat production after giving insulin.

METHODS

Wistar strain albino rats (200–250 g) of either sex were used. Many of the experiments were carried out on the conscious animal. In these experiments liver recorders were implanted under ether anaesthesia, usually on the previous day. The leads from the animals were soldered to the leads from the apparatus. No restraint other than the animal's usual cage was used, the leads from the apparatus hanging freely from above.

In other experiments the rats were anaesthetized with pentobarbitone sodium (Nembutal, Abbott Laboratories; 60 mg/kg intraperitoneally). Polythene cannulae were inserted into the trachea, a carotid artery, a femoral artery and, usually, an external jugular vein, after heparinization (Liquemin, Roche Products, 100 μg/kg intraperitoneally). Liver blood-flow recorders were inserted through a mid-line abdominal incision. Baboons (*Papio anubos*) were anaesthetized by ethyl chloride induction followed by open ether and intraperitoneal pentobarbitone sodium (75 mg/kg).

Liver blood flow. The technique of internal calorimetry fully described elsewhere (Birnie & Grayson, 1952; Grayson, 1952) was employed for assessment of liver blood flow. This technique depends on a thermo-electric measurement of liver thermal conductivity. It has been shown elsewhere (Grayson, 1952) that conductivity increment (i.e. thermal conductivity of living liver less the thermal conductivity of dead liver) is an approximately linear function of blood flow.

The simplified photographic recording method described by Grayson & Mendel (1957) was used, giving automatic temperature and conductivity readings at intervals of 41 sec. The cold junction was protected by a fine Polythene sleeve and situated in the abdomen alongside the portal vein. In all experiments the abdomen was closed with sutures. The only leads emerging from the animal were copper wire connexions to the galvanometer and heating circuit. Both thermojunctions and their constantan connexions were enclosed in the abdominal cavity and temperature records showed the difference in temperature between the heated thermocouple in the liver and the portal vein.

Blood pressure recording and regulation. In anaesthetized rats mean arterial blood pressure was recorded optically from a carotid artery, using a Wigger's capsule, and the blood pressure was prevented from changing during the course of the experiments by the methods already described, (Grayson & Johnson, 1953).

Estimation of blood glucose. Blood glucose was estimated by Nelson's photometric adaptation of the Somogyi method (Nelson, 1944).

Heat production in the liver. The method of estimating hepatic heat production used in the present work was based on an interpretation of the thermal conductivity record which is the basis of the technique of internal calorimetry for blood flow measurement. It involved no technical additions.

The remainder of the method is a matter of calculation from experimental results. Accordingly, it will be described, together with an experimental investigation of its validity, under Results.

RESULTS

The effect of insulin on liver blood flow in rats and baboons

Conscious rats. In ten experiments insulin (Insulin Soluble, 40 u./ml.; British Insulin Manufacturers) was given intraperitoneally to conscious rats, in which liver blood-flow recorders had been implanted, usually on the previous day. Fig. 1 shows the results of a typical experiment. It will be seen that blood flow was steady before and immediately after the administration of insulin. After 10 min blood flow, as indicated by the thermal conductivity, began to rise from resting conductivity increment (δk) levels of $5{\cdot}5\times10^{-4}$ to maximum levels of $10{\cdot}8\times10^{-4}$ after 55 min. Thereafter the flow fell slowly towards the level observed at the beginning of the experiment. Similar results were obtained in seven out of ten experiments of this kind. Flow increases began between 10 and 30 min after injection. Maximum flows were recorded between 45 and 80 min after injection. Returns to resting levels, where observed, were complete 1½–2 hr after injection.

Anaesthetized rats. In order to obtain records of changes in blood glucose, the experiments were repeated on anaesthetized rats. Fig. 2 shows the results of a typical experiment, performed on a rat in which the blood-flow recorder had been implanted on the previous day. Blood pressure was kept steady at 110 mm Hg by means of the compensator described previously. Samples for blood glucose estimation were withdrawn from the femoral artery.

After allowing a short period for stabilization, a sample of blood was withdrawn for glucose estimation, and insulin (2·0 u.) was given intravenously. There was a rapid rise in δk from resting levels of about $3{\cdot}4\times10^{-4}$ to maximum

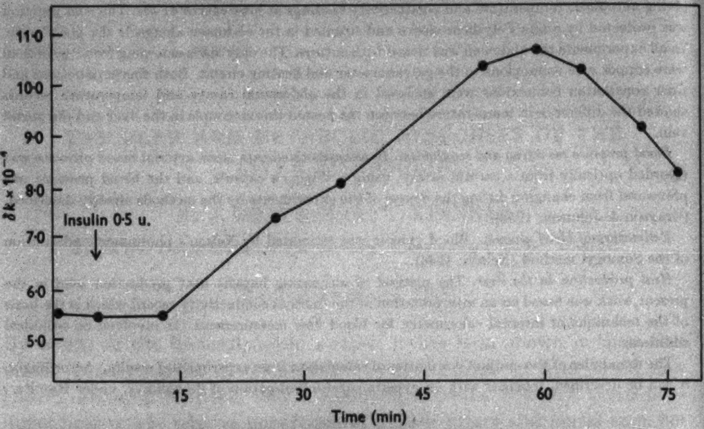

Fig. 1. The effect of intraperitoneal insulin (0·5 u.) on liver blood flow in the conscious rat. Blood flow in this and in other figures is given in terms of conductivity increment, δk.

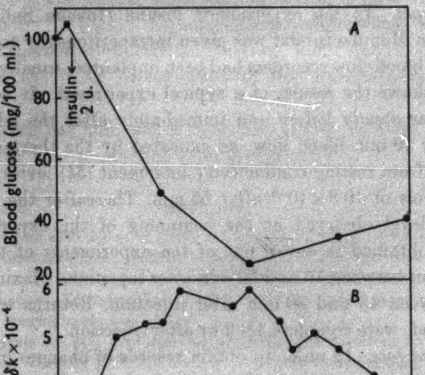

Fig. 2. The effect of intravenous insulin (2 u.); *A* on blood glucose and *B* on liver blood flow in the anaesthetized rat.

values of $5{\cdot}9 \times 10^{-4}$ after 45 min. The rise accompanied a fall in blood sugar from 100 to 25 mg/100 ml. After 90 min the liver blood flow had returned to initial levels, but the blood sugar showed only slight recovery. Similar results were obtained in eight out of ten experiments. The blood-flow change was similar in extent to that already described for the conscious rat, although more rapid in onset when insulin was given by the intravenous route.

Anaesthetized baboons. Two experiments were performed on baboons (*Papio anubos*), using a femoral artery for compensation of blood pressure and a femoral vein for adminstration of insulin and collection of blood samples. Fig. 3 shows the results obtained in one of these experiments. The rise in blood flow in both experiments was similar to that seen in the rat and in both cases accompanied a fall in the level of blood glucose. In one experiment where compensation was not used no change occurred in mean systemic blood pressure.

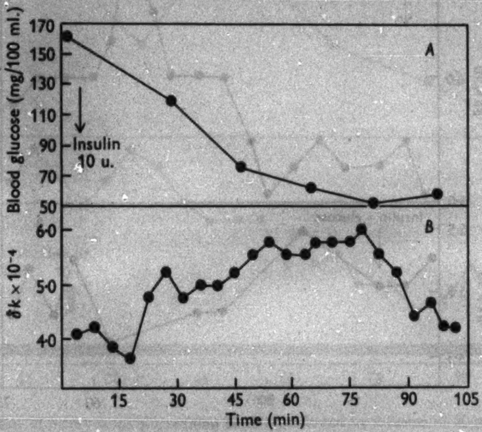

Fig. 3. The effect of intravenous insulin (10 u.); *A*, on blood glucose and *B*, on liver blood flow in the anaesthetized baboon.

The effect of insulin and glucose on liver blood flow

The conscious rat. Intraperitoneal injections of glucose alone were not observed to alter liver blood flow in the conscious rat. However, injections of glucose given before or at the same time as injections of insulin greatly modified the liver blood-flow responses. Fig. 4*A* shows the effects of insulin alone on a conscious rat. The expected increase in blood flow was recorded. Fig. 4*B* shows the results of an experiment performed on the same animal on the following day, when the same dose of insulin was combined with an injection of 100 mg of glucose. It will be seen that no significant rise in liver blood flow occurred. Similar results were obtained in three experiments.

The anaesthetized baboon. In two experiments on baboons the effects were investigated of glucose alone and of glucose in combination with insulin. Cannulae were inserted in both femoral veins, one femoral artery and the trachea. Blood pressure was compensated through the femoral artery. Blood samples were withdrawn from one femoral vein; the other vein was used for the administration of glucose. Liver blood-flow recorders were inserted via the mid line. In the experiment shown in Fig. 5, 5 g glucose was administered intravenously, followed by an infusion of glucose at the rate of 30 mg/min for 40 min. Ten minutes later the blood sugar level was about 180 mg/100 ml. Ten units of insulin were then administered intravenously. During the subsequent period of observation the blood sugar varied between 140 and 180 mg/100 ml. for a period of 35 min during which time there was a steady fall in liver blood flow.

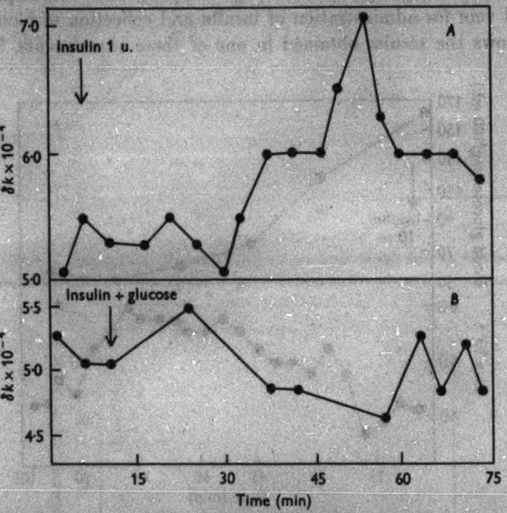

Fig. 4. *A*, the effect of intraperitoneal insulin (1 u.) on liver blood flow in the conscious rat. *B*, the effect of intraperitoneal insulin (1 u.) and glucose (100 mg) on liver blood flow in the same animal.

The anaesthetized rat. Two experiments were carried out in rats lightly anaesthetized with pentobarbitone sodium. Cannulae were inserted as previously described. Glucose was administered intravenously in dosage of 100 mg together with insulin in dosage of 1 u. Blood was withdrawn from the femoral artery for sampling and liver blood flow was recorded. Blood pressure was prevented from altering during the experiment by means of the compensator. As in the baboon experiments, in the presence of glucose no rise in liver blood flow occurred after insulin administration.

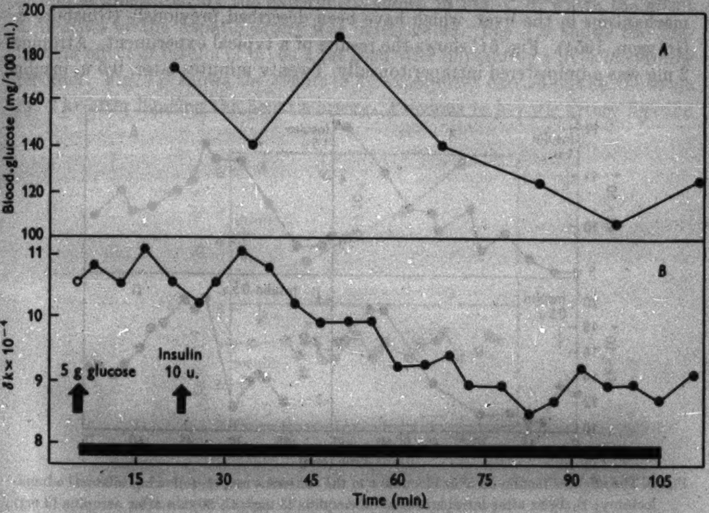

Fig. 5. The effect of intravenous glucose (5 g initially, followed by infusion at 30 mg/min during the period marked by horizontal bar) and intravenous insulin 10 u.; *A*, on blood glucose and *B*, on liver blood flow in the anaesthetized baboon.

The relation of nervous factors and the adrenal glands to liver blood-flow responses to insulin in the rat

Adrenalectomy. Four days before the experiment shown in Fig. 6*A* one adrenal gland was removed and a liver blood-flow recorder inserted; the other adrenal gland was removed 1 day before the experiment. It will be seen that following intraperitoneal injection of 1 u. insulin there was a rise in δk from resting levels of 9×10^{-4} to maximum levels of 12×10^{-4} in 70 min. Comparable results were obtained in two similar experiments.

Dibenzyline. The effect of the adrenolytic agent dibenzyline was investigated in three experiments in order to eliminate the possibility of other adrenergic mechanisms in the mediation of the hepatic blood-flow increase described previously. Fig. 6*B* shows the results of an experiment in which 3 mg of dibenzyline was administered intraperitoneally 1½ hr before commencing recording. 1·5 u. insulin was injected intraperitoneally. There was a rise in δk from resting levels of 3×10^{-4} to maximum levels of $4{\cdot}5 \times 10^{-4}$ 35 min after insulin administration. Similar results were obtained in the other experiments.

Atropine. The effect of atropine was investigated in three experiments in order to determine the possible involvement of cholinergic vasodilator mechanisms in the liver, which have been described previously (Ginsburg & Grayson, 1954). Fig. 6*C* shows the results of a typical experiment. Atropine 2 mg was administered intraperitoneally. Twenty minutes later, 0·5 u. insulin was injected intraperitoneally. (Larger doses of insulin were usually fatal following the administration of atropine.) There was a rapid rise in δk from resting levels of $12{\cdot}5 \times 10^{-4}$ to maximum levels of 19×10^{-4} 50 min after injection of insulin. Similar results were obtained in all experiments.

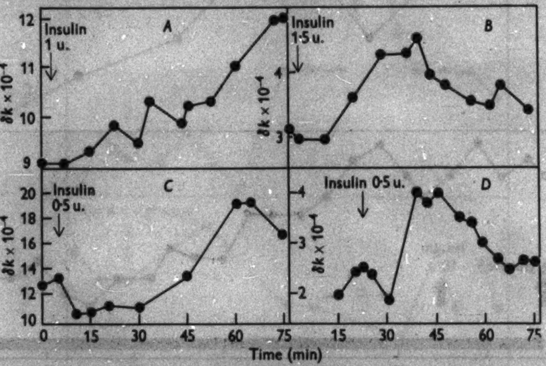

Fig. 6. The effect of insulin on liver blood flow in the conscious rat; *A*, following bilateral adrenalectomy; *B*, 1½ hr after intraperitoneal dibenzyline (3 mg); *C*, 20 min after atropine (2 mg) intraperitoneally; *D*, 20 min after intravenous tetraethylammonium bromide (10 mg). The insulin was given intraperitoneally in *A*, *B* and *C* and intravenously in *D*.

Ganglion blockade. The effect of tetraethylammonium bromide was investigated in order to elucidate the possible importance of other nervous mechanisms in the hepatic blood-flow response to insulin. In the experiment shown in Fig. 6*D*, 10 mg of tetraethylammonium bromide was administered intravenously. Twenty minutes later 0·5 u. of insulin was given intravenously (this dose was used for comparison with the effect noted after atropine). There was a rise in δk from resting levels of $2{\cdot}5 \times 10^{-4}$ to maximum levels of 4×10^{-4} 15 min after insulin. Similar results were obtained in four other experiments.

Coeliac neurectomy. In two sets of experiments coeliac neurectomy was carried out. In both cases insulin produced blood flow increments before and after the operation. Unfortunately, it was not possible to check the completeness of the nerve resection.

The role of the hepatic artery in the liver blood-flow response to insulin

The importance of the hepatic artery in the liver blood-flow response to insulin is illustrated by the experiment shown in Fig. 7, in which the effect of ligation of the hepatic artery was investigated. Fig. 7 *A* shows the preligation effect of insulin (1 u.) on a rat soon after implanting a liver blood-flow recorder. Fig. 7 *B* shows the results of an experiment carried out on the same rat 2 hr after ligating the hepatic artery. Previous to hepatic artery ligation δk levels in the liver rose from $3{\cdot}5 \times 10^{-4}$ to $6{\cdot}3 \times 10^{-4}$ following insulin. After hepatic artery ligation no rise in liver blood flow followed the injection of the same dose of insulin. Similar results were obtained in three experiments in which observations were made shortly after hepatic arterial ligation.

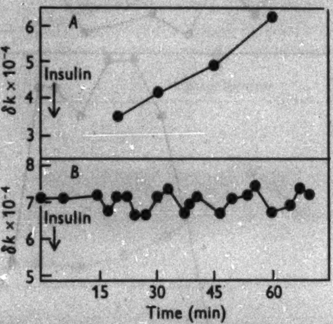

Fig. 7. The effect of intraperitoneal insulin (1 u.) on liver blood flow in the rat; *A*, before ligation of the hepatic artery; *B*, 2 hr after ligation of the hepatic artery.

In experiments which were carried out 24 hr and more after ligation of the hepatic artery, however, it was found that administration of insulin usually produced some rise in liver blood flow. In the experiment shown in Fig. 8 *A*, for example, 0·5 u. of insulin was given 2 hr after ligation of the hepatic artery, the animal having been permitted to recover from the anaesthetic. It will be seen that this dose of insulin failed to produce any significant change in liver blood flow. Twenty-four hours later, however, the same dose of insulin produced a marked increase in blood flow (Fig. 8 *B*).

Measurement of heat production in the liver

The use of heated thermocouples for the recording of thermal conductivity in the liver has suggested the possibility of measuring changes in intrahepatic heat production.

The temperature equilibrium in a heated thermocouple is governed by the formula $I^2 = F\theta k$, where I is the heating current, θ is the rise of temperature produced by the heating current, k is the thermal conductivity and F is the instrumental factor (Grayson & Haigh, 1954).

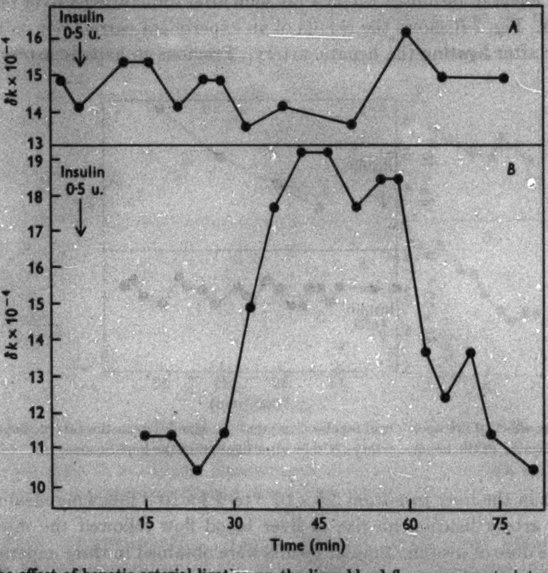

Fig. 8. The effect of hepatic arterial ligation on the liver blood flow response to intraperitoneal insulin (0·5 u.) in the conscious rat: *A*, 2 hr after ligation; *B*, 24 hr after ligation.

With the photographic method, temperatures are recorded from a thermocouple heated by a current of $0{\cdot}200\,\mathrm{A}^2$ and by a current of $0{\cdot}315\,\mathrm{A}^2$. The temperature increment, θ, produced by an increase in the heating current of $0{\cdot}115\,\mathrm{A}^2$ (i.e. from lower to upper equilibrium) may be measured directly from the record. From the formula $I^2 = F.\theta k$, θ is a linear function of I^2, provided k is constant. Thus, at any point on the graph of lower equilibrium temperatures, the temperature increment, t, produced by a current of $0{\cdot}200\,\mathrm{A}^2$ may be determined from the relation, $t = 0{\cdot}200/0{\cdot}115\theta$. This is the amount by which the lower equilibrium heating current raises the liver temperature under the conditions of thermal conductivity, and hence of blood flow appertaining at that point.

This calculation assumes that no other factor apart from blood flow affects the temperature recording. Clearly, however, several other circumstances, among which is local heat production, may produce a discrepancy between calculated and actual temperature of the lower equilibrium.

These factors were investigated:

Generalized change in the temperature of the animal. The internal temperatures of a rat, particularly an anaesthetized rat, undergo change in response to alterations in environmental temperature, heat elimination, heat production or from other general causes. The effect of changes of this nature was eliminated in the present experiments by placing the cold junction in the abdomen near the main blood supply of the liver, so that both cold junction and recording junction were subjected to the same variations in over-all temperature. The validity of this procedure is confirmed by experiments such as that shown in Fig. 9 A, where absolute temperatures were recorded from the liver and from near the portal vein. The animal was placed in a warm environment at 37° C. It will be seen that both liver temperature and temperature near the portal vein responded similarly.

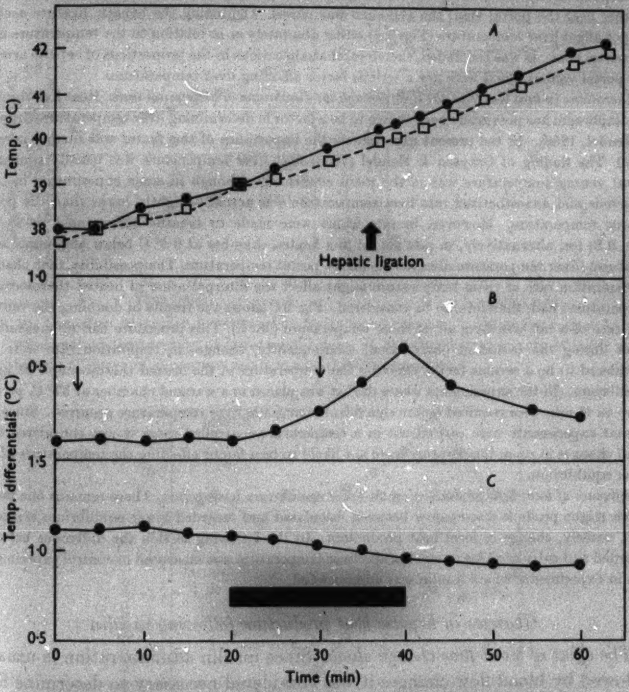

Fig. 9 *A*. The effect of hepatic arterial ligation on liver temperature (●) and portal vein temperature (□). Anaesthetized rat in warm chamber at 37° C. *B*. The effect of breathing warm air through a tracheotomy tube, during period between arrows, on liver–portal vein temperature differential; anaesthetized rat. *C*. The effect of increasing respiration rate from 65 to 110 c/min during period marked by horizontal bar. Animal artificially respired and exposed to room at 28° C; stroke volume constant throughout; anaesthetized rat.

Alterations in the proportion of hepatic arterial and portal venous blood reaching the liver. The possibility of error from the double blood supply was suggested by the observations of Federov & Shur (1942) who showed that the temperature of the aorta in the dog was 1° C higher than that of the portal vein. However, in the rat the hepatic artery is closely approximated to the portal vein for much of its course and it seems unlikely that a differential of this order could occur. To eliminate the possibility definitely, however, in three experiments a ligature was placed loosely around the hepatic artery at open operation. A simple thermocouple was inserted into the liver and another near the portal vein; the abdomen was closed. Tightening the hepatic ligature acutely did not affect liver temperature (Fig. 9*A*) either absolutely or in relation to the temperature near the portal vein. It was concluded, therefore, that alterations in the proportions of hepatic arterial and portal venous blood were not a serious factor affecting liver temperature.

Alterations in heat loss from the liver through the diaphragm or abdominal wall. Heat loss through the diaphragm has previously been shown to be a factor in determining liver temperature (Grayson & Mendel, 1956). In the present experiments the importance of this factor was further investigated. The finding of Grayson & Mendel (1956) that liver temperature was usually similar to portal venous temperature was in the main confirmed, although in some experiments in both conscious and anaesthetized rats liver temperature was actually slightly lower than the portal venous temperature. However, in rats which were made to breathe air warmed to 38·5° C (Fig. 9*B*) (or, alternatively, in rats placed in a heated chamber at 0·5° C below abdominal temperature), liver temperature always rose above portal temperature. The possibility that changes in respiration rate or room temperature might affect the interpretation of heated thermocouple temperatures had, therefore, to be considered. Fig. 9*C* shows the results of doubling the ventilation rate of a rat breathing air at room temperature (28° C). This procedure had no measurable effect during the period of observation. Consequently, changes in respiration rate were not considered to be a serious factor affecting the temperature of the heated thermocouple at lower equilibrium. In the experiments where the rat was placed in a warmed chamber at 37° C, periods of up to 45 min were required before significant changes in liver temperature occurred. Since the present experiments were carried out in a temperature-controlled room it was considered that small changes in room temperature were not likely to be a factor affecting the temperature of the lower equilibrium.

Influence of local heat production on the lower equilibrium temperature. There remains one factor which might produce discrepancy between calculated and recorded lower equilibrium temperature, namely, change in local heat production. In the following section the difference between recorded and calculated lower equilibrium liver temperature was measured in control experiments and in experiments where insulin was administered.

Changes in hepatic heat production following insulin

The effect of blood-flow change alone. Since insulin administration is usually followed by blood-flow changes it was considered necessary to determine first the effect of blood-flow change on observed and calculated lower equilibrium temperatures.

Fig. 10 shows the results of an experiment on a baboon in which blood-flow alterations occurred as a result of a drop in blood pressure from 60 to 35 mm Hg. No insulin was given. Fig. 10*D* shows the drop in blood flow which occurred in the liver. Fig. 10*A* gives the actual temperature of the lower equilibrium, Fig. 10*B* the calculated temperature. Fig. 10*C*, the curve of excess temperature, is a plot of the difference. It will be seen that actual and calculated temperatures were similar and that the curve of excess temperatures was not affected by the blood-flow change.

The effect of insulin on the curve of excess temperature. Fig. 11 gives the results of three typical experiments in which blood flow and excess temperature were recorded following the administration of insulin. The blood-flow responses are not shown but in each case were similar to those previously described. It will be seen that in all three experiments there was a rise in lower equilibrium

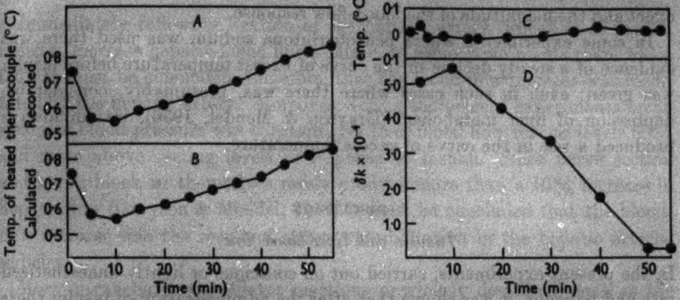

Fig. 10. The effect of sudden lowering of systemic blood pressure in the anaesthetized baboon, on *A*, actual temperature of lower equilibrium; *B*, calculated temperature of lower equilibrium; *C*, curve of excess temperature and *D*, liver blood flow.

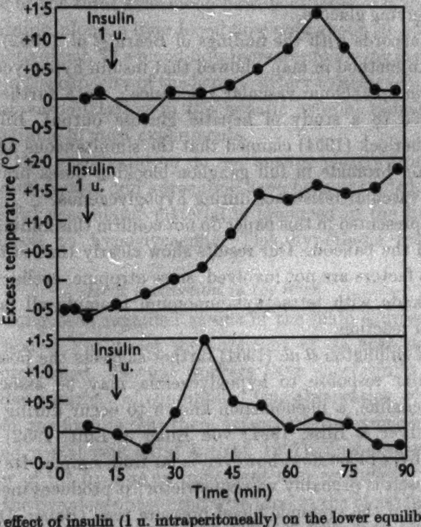

Fig. 11. The effect of insulin (1 u. intraperitoneally) on the lower equilibrium excess temperature curve in three anaesthetized rats.

temperature, after insulin administration, greater than could be accounted for on the basis of altered blood-flow. The rise in each instance either coincided with or slightly preceded the rise in blood flow. This effect has been a consistent finding. Even in the few instances where blood-flow changes were minimal, the excess temperature curve always showed a marked rise. It has not been possible to demonstrate any correlation between the extent of this effect and the magnitude of the blood-flow response.

In some experiments where pentobarbitone sodium was used there was evidence of a steady decline in the curve of excess temperature before insulin was given; even in such cases where there was, presumably, considerable depression of liver metabolism (Grayson & Mendel, 1956), insulin always produced a rise in the curve of excess temperature.

DISCUSSION

Insulin and liver blood flow

In the present experiments, carried out on conscious or lightly anaesthetized rats and baboons, it was shown that after the administration of insulin there was a marked increase in liver blood flow as measured by the technique of internal calorimetry. The rise in liver blood flow coincided with a substantial reduction in the level of blood glucose and did not occur if hypoglycaemia was prevented by giving glucose.

This result accords with the findings of Bearn *et al.* (1952) who, using the bromsulphalein method in man, showed that insulin hypoglycaemia produced a 'slight fall in splanchnic vascular resistance'. In a further investigation, mainly directed to a study of hepatic glucose output, Billington, Paton, Reynolds & Sherlock (1954) claimed that the simultaneous administration of hexamethonium bromide in full ganglion-blocking doses prevented this fall in splanchnic vascular resistance during hypoglycaemia.

The results presented in this paper do not confirm this finding with reference to the rat and the baboon. Our results show clearly that in these species, at least, nervous factors are not involved, since atropine, coeliac neurectomy or ganglion blockade with tetraethylammonium bromide all failed to modify the vasodilator reaction.

The work of Billington *et al.* (1954) further suggests the possibility that the hepatic vascular response to hypoglycaemia may be associated with the release of adrenaline, a phenomenon known to occur during hypoglycaemia (Cannon, MacIver & Bliss, 1924; von Euler & Luft, 1952). The action of adrenaline on liver blood flow, however, is far from simple. Its direct action on liver blood vessels is probably vasoconstrictor; it produces increase in hepatic blood flow only if the systemic blood pressure rises (Bauer, Dale, Poulsson & Richards, 1932; Grayson & Johnson, 1953). In our experiments, during insulin hypoglycaemia, mean systemic blood pressure remained constant. Moreover, following bilateral adrenalectomy or the administration of the adrenolytic agent dibenzyline, in adequate blocking dosage, marked increases in liver blood flow still occurred during hypoglycaemia. It must be concluded, then, that although adrenaline release is an important feature of insulin hypoglycaemia, it is not the factor responsible for the increase in liver blood flow.

Immediately following ligation of the hepatic artery, injection of insulin failed to increase hepatic blood flow; indeed, in some experiments there was a fall. It must be concluded, therefore, that the blood-flow increase was meditated by the hepatic artery. Moreover, during the present experiments mean systemic blood pressure was constant, yet liver blood flow rose to levels 60% and more above resting levels in response to insulin. Since nerve section and nerve block in themselves rarely produce more than a 10% increase in hepatic flow (Grayson & Mendel, 1956) it must be concluded that the blood-flow increase was the result of active vasodilatation in the hepatic arterial radicles.

Most intrahepatic vasodilator reactions previously described, such as the response to raised blood pressure (Ginsburg & Grayson 1954), have been transient and small relative to the effects described in this paper; but it should be remembered that the hepatic artery, although normally carrying only a small percentage of the total liver blood flow, is capable (e.g. during haemorrhage) of maintaining, alone, a liver blood flow only about 30–40% below that found in the intact animal. The blood-flow increases following insulin are therefore clearly within the capacity of the hepatic artery to achieve unaided. Even so, the degree of vasodilatation in the hepatic arterial radicles must have been very considerable in most experiments.

The causes of this vasodilatation do not emerge clearly from the present work, since nervous factors and adrenaline have been excluded. It is possible that some powerful vasodilator material is produced in the liver during its metabolic responses to hypoglycaemia. This hypothesis requires further exploration.

Heat production in the liver

The liver has long been regarded as one of the main sources of heat production in the body. Yet direct measurement of its heat production has never been accomplished. Several workers have successfully measured its temperature under a variety of conditions. One observation which has given difficulty has been the finding that there exists little difference in temperature between liver and portal vein (Graf & Graf, 1957). This does not mean that the classical view of the liver as an important heat producer is wrong, but it does imply that the liver loses heat otherwise than to the blood flowing through it. The present experiments show that some of this heat loss takes place through the diaphragm and that abolition of temperature gradients across the diaphragm causes the liver to register a substantially higher temperature than that of the portal blood.

Nevertheless, although heat loss across the diaphragm can be demonstrated, it has, thus far, proved impossible to estimate its extent. Simple calculation of heat production from simultaneous measurements of blood flow and liver-portal temperature differences is, therefore, impossible.

In the present work the temperature of the lower equilibrium of the heated thermocouple was used as a basis for measurement. By means which have already been described in detail it was possible to determine the temperature changes which could be ascribed to changes in blood flow. The influence of such factors as variations in internal temperature of the animal, uneven mixing of hepatic and portal blood, and heat loss through the diaphragm, have been considered in full. The conclusion that artifacts of this kind do not affect the curve of excess temperature is further supported by the experiments in which blood-flow changes were induced by blood-pressure change alone; in these, calculated and actual lower equilibrium temperatures were the same. In consequence it seems justifiable to regard the divergence of calculated and recorded temperatures which occurred following insulin as being, in fact, due to change in local heat production in the liver.

Insulin and heat production in the liver

In the present experiments it was shown that following insulin administration the temperature of the lower equilibrium of the heated thermocouple rose by an amount which could not be accounted for by blood-flow change. The effect was consistent. It accompanied or preceded the rise in blood flow. In view of the evidence already adduced, the results can only be interpreted as being due to increased intrahepatic heat production, a metabolic sequel to the administration of insulin. In some experiments there was evidence of considerable depression of heat production at the beginning of the experiments, presumably the result of pentobarbitone sodium anaesthesia. Even in these circumstances, insulin hypoglycaemia could still evoke increased heat production.

These results can only be regarded as preliminary. They demonstrate, however, the practicability of recording certain metabolic events within the liver.

SUMMARY

1. The effect of insulin on liver blood flow measured by the method of internal calorimetry was investigated in the rat and the baboon.

2. A method of estimating changes in heat production in the liver from the thermal conductivity record is described.

3. Marked increases in liver blood flow occurred during insulin hypoglycaemia, and could be prevented by the administration of glucose.

4. The blood-flow response was not modified by bilateral adrenalectomy, or coeliac neurectomy or by the administration of atropine, tetraethylammonium bromide or dibenzyline. Nervous factors and adrenaline release were, therefore, not responsible for the rise in liver blood flow.

5. After hepatic arterial ligation no increase in flow followed insulin administration. The vasodilatation was thus confined to the hepatic arterial radicles.

6. After insulin administration there was a rise in hepatic heat production, which corresponded in time to the increase in liver blood flow.

7. It is suggested that the metabolic response of the liver to hypoglycaemia may be accompanied by the liberation of a local vasodilator substance in the liver.

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PLAIN-MUSCLE STIMULANTS IN EXTRACTS OF MENSTRUAL FLUID AND OF ENDOMETRIAL CURETTINGS

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The myometrium contracts rhythmically during menstruation. This activity may not only expel the decidua menstrualis as it is sloughed off, but it may help the process of sloughing by intermittently altering the volume of the vessels that lead to the endometrial spiral arteries (Reynolds, 1949). It is not known how it is stimulated or controlled.

In preliminary communications (Pickles, 1957; Chambers & Pickles, 1958) it has been suggested that the menstrual contractions of the myometrium may be caused by a plain-muscle stimulant, liberated by the endometrium and passing to the myometrium; and evidence has been outlined that such a substance is present in extracts of menstrual fluid and of endometrial curettings. This paper gives the evidence in detail.

METHODS

Extractions

Menstrual fluid. The subjects were healthy students. Under the direction of a woman colleague they were provided with vessels containing 20 ml. of acetone, into which the vaginal tampons used during the first 2 days of the menstrual period were put immediately they were discarded. The method of extraction was essentially that described previously (Pickles, 1957); in detail, the procedure was as follows. The tampons from each subject were first pounded with 100 ml. of acetone at room temperature; but as the menstrual fluid contained water, these first extractions were in effect made with acetone containing on a rough estimate about 25 % of water. The tampons were further eluted with another 100 ml. of acetone, then with 100 ml. of diethyl ether and finally with 100 ml. of petroleum ether (boiling range 60–70° C). These extracts were filtered and combined and the solvents were evaporated off under reduced pressure at a temperature of not more than 40° C. The resulting yellow-brown solid or very viscous liquid is referred to as the 'total lipids', although it is not claimed either that all the menstrual constituents that might be called lipids were present, or that other substances were absent.

In most experiments the 'total lipids' were dissolved as far as possible in either anhydrous acetone or ether, or in a few cases in chloroform. The solution was filtered and the filtrate was added to Locke's solution that had been brought to about pH 10 with NaOH, the organic solvent then being evaporated off and the solution neutralized with HCl. In some experiments the 'total lipids' were fractionated by differential elution from a silicic acid column as described by Fillerup & Mead (1953) and the final solutions were dissolved or suspended in Locke's solution in the way described above, to a concentration of 1 mg of lipid/ml. The more lipophilic fractions were only slightly soluble in Locke's solution, but formed suspensions which were well shaken before being used.

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Several experiments on the partition of the active substances between ether and Locke's solution at different hydrogen ion concentrations were done as follows. A saturated solution in ether of the ether-soluble part of the 'total-lipids', or of the most hydrophilic fraction prepared by chromatography as described above, was filtered and then shaken with several times its own volume of Locke's solution brought to pH 3 with HCl; this was run off, replaced by Locke's solution brought to pH 10 with NaOH, and the partition repeated. The Locke's solutions were filtered and neutralized. The first was likely to contain the more basic lipids and a greater part of those whose partition between ether and water was unaffected by pH; the second would contain the more acid lipids.

Control extracts of unused tampons were made by the same method as that described for the total lipids of menstrual fluid. These controls were inactive. A further control extract was made by absorbing on vaginal tampons 20 ml. of heparinized venous blood, taken on the 8th day of the cycle from the forearm of a subject whose menstrual fluid had previously been shown to contain the active principle, and then eluting the tampons in the usual way.

Endometrial curettings. Immediately after operation the curettings were collected into polythene or siliconed glass vessels, and rinsed with 0·9% (w/v) saline. Either at once or after being stored at −10° C for not more than 2 days, they were extracted as follows. They were ground in a glass tissue-grinder with acetone, and extracted with acetone, ether and petroleum ether in the same way as were the menstrual specimens. The solvents were evaporated off at a temperature not above 37° C under decreased pressure, leaving a light yellow solid or very viscous liquid, referred to as the endometrial 'total lipids'. These were treated in the same ways as were the menstrual 'total lipids'.

To test the possibilities that an active principle was formed by contact with glass or with organic solvents, one lot of curettings was crushed in a polythene vessel with a 'Perspex' (plastic) rod in the presence of alkaline Locke's solution at pH 11. This solution was filtered, neutralized and used without at any time coming into contact with glass.

Isolated tissue preparations

The guinea-pig jejunum and strips of human myometrium were most frequently used. Pieces of uterus were brought from the hospital immediately after hysterectomy, and strips were cut as shown in Fig. 1, and suspended without delay. Other tissues sometimes used were the guinea-pig seminal vesicle, slit lengthwise and washed clear of its contents, and the rat vas deferens and colon.

All preparations except those of the rat were suspended in oxygenated Locke's solution made from stock solutions as described by Umbreit, Burris & Stauffer (1945), in the following proportions: NaCl 0·90% (w/v) 100 parts, KCl 1·15% (w/v) 2 parts, $CaCl_2$ 1·22% (w/v) 4 parts, $NaHCO_3$ 1·3% (w/v) 2 parts, glucose 2 g/l. of the final solution. For the rat colon and vas deferens the special rat-colon solution described by Gaddum, Peart & Vogt (1949) was used. If the spontaneous activity of the guinea-pig tissues proved troublesome, Mg in the form of 154 mM-$MgSO_4$ solution was added in the smallest effective quantity, usually 10–20 ml./l. In many experiments the bath fluid contained atropine, mepyramine and bromolysergic acid in concentrations of $1–2 \times 10^{-7}$ g/ml.

The organ baths had a volume of 10 ml. and the levers were arranged to magnify the contractions tenfold. The initial length of each tissue was marked on the tracing, so that contractions could be expressed as percentage changes of length. The load on the lever was adjusted to 0·2 g wt. at the 'relaxed' length of the tissue, and this load increased slightly as the preparations contracted.

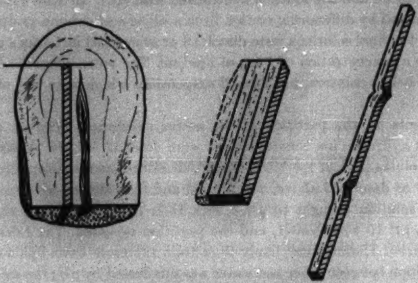

Fig. 1. The method of cutting the strip of human myometrium. The final strip is about 4 cm long and 1 mm^2 in cross-section.

RESULTS

Depending on the kind of plain-muscle preparation and the dose of the extract used, the following kinds of response were found, singly or combined:

(1) a simple contraction, which was characteristically 'slow', taking from ½ to 5 min to reach its peak;

(2) initiation of rhythmical contractions, or their enhancement or alteration in their rhythm if they were already present;

(3) potentiation of other stimulants such as acetylcholine applied later.

The effects persisted for varying times after the extracts had been washed out of the perfusing fluid. They were given alike by acetone or ether extracts of either endometrial or menstrual lipids, and by the few chloroform extracts that were made of menstrual lipids.

The responses of various tissues

Human uterus. The response varied according to the amount of spontaneous rhythmical activity shown by the preparation. When the spontaneous activity was small the extracts commonly caused a moderately slow contraction which was not maintained even when the extract was kept in the bath (Fig. 2). If, however, the preparation was already showing pronounced rhythmical contractions, the extent of these contractions at their peaks was always increased and the increase was maintained for at least several minutes while the extract was kept in the bath. In some such instances the muscle relaxed between the contractions in the presence of the extract almost as well as in its absence, so that the amplitude of the contractions was increased; an example of this kind of response is shown in Fig. 3. In other instances relaxation between contractions was incomplete in the presence of the extract, so that the amplitude of the contractions was increased only slightly or was even decreased. Whatever form the stimulation took, it was not inhibited by atropine sulphate, mepyramine maleate, or bromolysergic acid diethylamide tartrate, either separately or together in a concentration of 2×10^{-7} each.

No potentiation of the stimulant action of vasopressin on the human uterus was seen, although several attempts were made to demonstrate such an action of the menstrual extracts. The possible effects on the action of other uterine stimulants were not investigated.

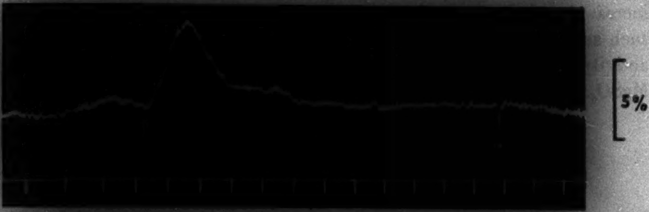

Fig. 2. Effect of menstrual extract on a strip of human myometrium (patient aged 43) suspended in 10 ml. Locke's solution at 36° C. At the arrow 0·3 mg of ether extract of menstrual lipid was added, and at the white dot the extract was washed out. In this and the following figures the vertical line corresponds to a 5% change in the length of the preparation and the time marks show intervals of 1 min.

Fig. 3. Effect of menstrual extract on a strip of human myometrium (patient aged 44) suspended in 10 ml. Locke's solution at 37° C containing atropine sulphate, mepyramine maleate and bromolysergic acid diethylamide tartrate, each 2×10^{-7} g/l. At the arrow, 0·2 mg of ether extract of menstrual 'total lipids' added to the bath; at the white dot the extract was washed out.

Guinea-pig small intestine. The extracts caused a slow contraction, commonly preceded by a latent period of a few seconds, and taking 0·5–5 min to reach its peak (see Fig. 4). If the extract was left in the bath, the intestine partially relaxed, and often then slowly contracted and relaxed again rhythmically on a base line higher on the tracing than the resting level. The increased tone and slow rhythmical contractions commonly persisted for several minutes after the extract had been washed out.

The responses were not inhibited by the presence of atropine sulphate, mepyramine maleate, or lysergic or bromolysergic acid diethylamide tartrate, either separately or together in a concentration of 2×10^{-7} g/ml. each. Addition of $MgSO_4$ to give concentrations of more than 3 mM diminished the responses, and inhibited any activity persisting after the extract had been washed out. For example, in the experiment of Fig. 4 the intestinal preparation relaxed slowly and incompletely when the extract was washed out, but when $MgSO_4$ was added to the bath the relaxation was accelerated and the rhythmical contractions ceased. When the $MgSO_4$ was also washed out, the tone increased and rhythmical activity reappeared, although there had been little such activity before the menstrual extract was applied. Both the rhythmical activity and the increase in tone were abolished by further addition of $MgSO_4$.

Fig. 4. Effects of menstrual extract and of Mg on a piece of guinea-pig small intestine, suspended in 10 ml. Locke's solution at 36° C containing atropine sulphate, mepyramine maleate and bromolysergic acid diethylamide tartrate, each 2×10^{-7} g/l. At *A* 0·3 mg of ether extract of menstrual lipid was added; at the two white dots, the preparation was washed. At *B* and *C* isotonic $MgSO_4$ solution was added, to give a concentration first of 4·6 mM and then of 10·7 mM. The preparation was next passively stretched, and washed where shown by the white dots. At *D*, $MgSO_4$ was added to give a concentration of 3·1 mM.

Amounts of the extracts that were alone too small to cause any direct contraction of the intestinal muscle often but not always increased the responses to acetylcholine, histamine or 5-hydroxytryptamine, added to the bath 1–3 min later. Such a potentiation of the effect of acetylcholine is shown in Fig. 5.

Guinea-pig seminal vesicle. The responses varied. Some preparations responded with a slow contraction; others with a slow increase in their rhythmical activity, as shown in Fig. 6. Other preparations gave no direct response to quantities of total lipids of up to 1 mg in the 10 ml. bath, but in some of these the extracts increased the responses to acetylcholine, noradrenaline or adrenaline, all of which cause contraction. The cause of this different behaviour of different preparations is unknown.

Rat vas deferens and colon. Only menstrual extracts were tested on these tissues, as the amounts of endometrial extracts available were too small. On the vas deferens, the menstrual extracts never directly caused any contractions, but they increased those caused by subsequent doses of adrenaline or noradrenaline. This is shown for noradrenaline in Fig. 7. On the rat colon the extracts regularly caused a slow increase in tone, on which were superimposed frequent large contractions. This is illustrated in Fig. 8*b*, which shows the effect of 0·2 mg of each of two chromatographic fractions (see p. 76) of menstrual lipids. If the colon preparation showed some large contractions before the addition of the extract, these continued at a greater frequency when the extract was added.

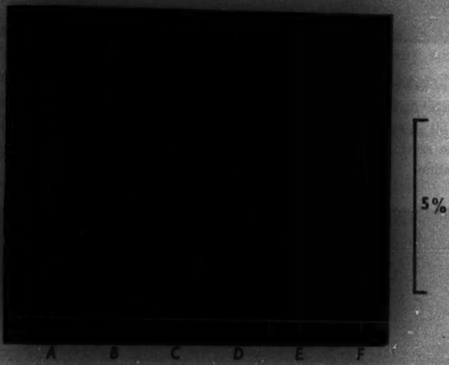

Fig. 5. Guinea-pig small intestine suspended in 10 ml. Locke's solution at 33° C containing $MgSO_4$ 3·1 mm. Potentiation of the effect of ACh by menstrual extract with little or no direct stimulant action. The contractions are responses to 0·5 ng ACh applied for 15 sec every 2 min, except for *F* in which the dose was 1·0 ng. For 1 min before *B* 20 μg, and before *D*, 30 μg, of chromatographic fraction 5 of menstrual lipid was added, as indicated by the arrows.

Fig. 6. Effect of endometrial lipid extract (*A*) on the activity of a guinea-pig seminal vesicle suspended in 10 ml. Locke's solution plus $MgSO_4$ 7·7 mm. This quantity of $MgSO_4$ was just enough to inhibit most of the activity of the preparation in the absence of endometrial lipid. Extract washed out where shown by white dot.

Fig. 7. Rat vas deferens suspended in 10 ml. modified Locke's solution plus $MgSO_4$ 2·3 mM; responses to 30 μg noradrenaline bitartrate applied for 40 sec every 2 min, except that at *a* the dose was 50 μg and at *b* it was 70 μg. *A*, ether extract of menstrual lipids applied for 1 min before the next test with noradrenaline; *B*, the same quantity applied along with the noradrenaline.

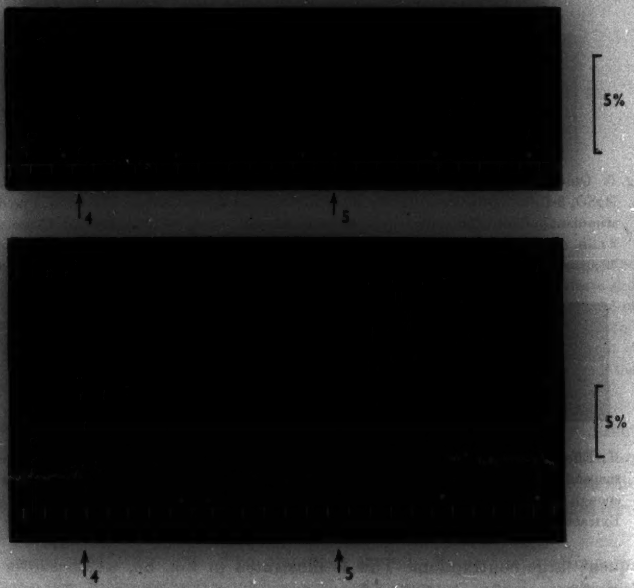

Fig. 8. Effects of chromatographic fractions 4 and 5 of menstrual lipids (*a*) in potentiating the effect of noradrenaline on the rat vas deferens and (*b*) in directly stimulating contractions of the descending colon from the same rat. Each preparation suspended in 10 ml. modified Locke's solution. At *n* the vas deferens received each time 40 μg of noradrenaline bitartrate. At (4) the vas was given 0·4 mg and the colon 0·2 mg of chromatographic fraction No. 4 of menstrual lipids; at (5) the same quantities of chromatographic fraction 5 were given. Preparations washed where shown by white dots.

The general nature of the active principle

No consistent difference between the effects of similar extracts of menstrual fluid and of endometrial curettings has yet been found, apart from a possible instance with one chromatographic fraction (see p. 77). The effects produced by extracts of menstrual fluid or endometrial curettings could not be attributed to histamine, acetylcholine, or 5-hydroxytryptamine, since they were not inhibited by atropine, mepyramine or bromolysergic acid, and the contractions were much slower in their time course than those usually resulting from these known stimulants. On the other hand, histamine-like activity which was inhibited by mepyramine was found in the residues left after the extraction of menstrual fluid with organic solvents, and therefore histamine may well be a constitutent of menstrual fluid.

The effects could not be attributed to potassium. The K content of an acetone extract of menstrual fluid, estimated by flame photometry, was found to be less than 1/100 of the minimum necessary to produce effects like those obtained with the extract. Nor could the effects be attributed to a polypeptide or to any other substance formed by contact with glass or by the action of the organic solvents. An aqueous extract of curettings, prepared without contact with glass and tested on a guinea-pig small intestine in a freshly siliconed organ bath in the presence of atropine, mepyramine and bromolysergic acid (2×10^{-7} each), gave responses indistinguishable from those of extracts prepared in the usual way. The fact that not only extracts prepared with acetone were active, but also those prepared with sodium-dried ether or chloroform, makes it unlikely that the effects were due to a polypeptide.

In partition between ether and aqueous solutions at different pH, the characteristic activity appeared predominantly in the fractions likely to contain the acids.

The blood which is part of the menstrual fluid or which contaminates the endometrial curettings is not the source of the substance or substances responsible for the effects on plain muscle, since an extract of venous blood treated in the same way as the menstrual fluid showed, in similar quantities, no detectable activity. The amount of lipid extracted from this specimen of venous blood was 520 mg/100 ml., which is within the normal range. In the experiment illustrated in Fig. 9, the human uterine preparation showed some spontaneous contractions, and the only effect obtained with 1 mg of lipid from venous blood was that these became perhaps less frequent and less pronounced. When however 0·3 mg of lipid from menstrual fluid was added, there occurred the typical slow rise in tone upon which large contractions were superimposed.

Chromatographic fractionation

When the 'total lipids' of either menstrual fluid or endometrial curettings were fractionated by elution from a silicic acid column with an increasingly hydrophilic series of solvents, and tested on the human uterus, the guinea-pig small intestine and the rat vas deferens and colon, the following results were obtained. Fractions 1 and 2, eluted with 1 and 4% diethyl ether in petroleum ether, respectively, showed little or no activity either directly stimulant or potentiating. Fraction 3 of the endometrial lipids, eluted with 10% ether in petroleum ether, likewise showed no activity; but the corresponding fraction

Fig. 9. Strip of human uterus (patient aged 34) suspended in 10 ml. Locke's solution At (*a*) effect of 1 mg of total lipids extracted from venous plasma of a subject aged 37; at (*b*) effect of 0·3 mg of total lipids from menstrual fluid of the same subject.

Fig. 10. Effect of 0·1 mg of chromatographic fraction 5 of menstrual lipid on a strip of human uterus (patient aged 34); lipid added where shown by arrow and washed out where shown by white dot.

of menstrual lipids caused a slow contraction of the guinea-pig small intestine, the human uterus and the rat colon. This effect, however, was not always obtained. Fraction 4, eluted with 50% ether in petroleum ether, showed the greatest potentiating activity; whereas fraction 5, eluted with 25% methanol in ether, showed the greatest directly-stimulating activity. In Fig. 10 the strong stimulating effect of 0·1 mg of fraction 5 on the human uterus preparation is seen. In the experiment of Fig. 8, when fractions 4 and 5 were compared on the rat preparations, fraction 5 had the greater directly stimulating action on the colon, whereas fraction 4 had the greater potentiating effect on the vas deferens. Fraction 4 was also found to be more effective than fraction 5 in potentiating the effect of noradrenaline on the guinea-pig seminal vesicle, whereas fraction 5 had the greater directly-stimulating effect. From this it seems that the extracts contain at least two active substances, which can be partially separated by chromatography.

Quantitative comparison of different extracts

The amount of 'total lipids' extractable from a menstrual specimen, probably containing the greater part of one discharge in one subject, was about 100 mg, but varied widely. The specimens were not usually weighed before extraction, but in those that were the 'total lipids' represented about 0·3% of the original weight of the menstrual fluid. In the chromatographic fractionation, the fifth and most active fraction contained about one-quarter of the total weight of lipid.

One preparation of this fraction was used as a provisional standard. It was derived from two menstrual specimens from one subject, and contained a total of 43 mg of lipid. An effective dose on most tissues was 0·1–0·2 mg in a 10 ml. organ bath, but a sensitive guinea-pig small intestine would sometimes respond to 0·01 mg.

The curettage specimens, weighed moist after being rinsed with saline and drained, averaged about 1 g per subject, and the 'total lipids' were about 1% of this weight. The amounts of lipids which produced effects on the guinea-pig intestine and the human uterus were the same as those of lipid from menstrual fluid.

DISCUSSION

The evidence given shows that the endometrium and menstrual fluid alike contain one or more lipids that stimulate plain muscle. Although the presence of common stimulants of plain muscle in these sources is not excluded, and indeed the menstrual fluid has been shown to contain some histamine-like substance, the typically slow and long-acting effects are not attributable to such substances. Nor can they be attributed to the method of preparation of the extracts, or to the presence of blood in the menstrual fluid or curettings.

The properties of some lipoid stimulants of plain muscle have been reviewed by W. Vogt (1957). Other substances of the same general nature include 'eutocin' extracted from human liquor amnii during labour (Hanon, Coquoin-Carnot & Pignard, 1953); and a substance extracted from the placenta of the guinea-pig (Cantoni & Martini, 1955). The chemical properties of most of those substances remain largely unknown, and the same is true of the active substances in the menstrual fluid and endometrial curettings. It is therefore impossible to say yet with certainty whether the latter substances are identical with some substances already named, and for this reason no names are proposed for them at present.

Although the physiological significance of the active substances is at present conjectural, it is reasonable to suppose that they may escape to some extent into the circulating blood during menstruation, and by stimulating the myometrial muscle may help the expulsion of the decidua menstrualis. They may perhaps also provide an explanation of the raised gastro-intestinal activity during menstruation, and the occurrence of colic or diarrhoea in some breast-fed babies during the mother's menstrual periods (Moncrieff & Thompson, 1952; Fochem, 1955).

SUMMARY

1. The lipids of human menstrual fluid and of endometrial curettings have been extracted by means of organic solvents, transferred to Locke's solution, and tested on various plain-muscle preparations.

2. Both menstrual and endometrial lipids produce a slow contraction of plain muscle in several different kinds of preparation, particularly the human uterus and the guinea-pig jejunum.

3. Evidence is given that this effect is not due to any of several known stimulants of plain muscle.

4. The extracts of menstrual and endometrial lipids potentiate other stimulants of plain muscle. In some instances this effect can be shown without any direct stimulation of the muscle.

5. After partition of the lipids between aqueous solutions of different pH and ether, the greater part of the activity is contained in the fractions that are likely to contain the acid substances.

6. The lipids can be separated chromatographically into (*a*) an inactive fraction, (*b*) a fraction with a high power of potentiating other stimulants but a low power of direct stimulation, and (*c*) a fraction with relatively low potentiating power but a high power of direct stimulation.

7. It is suggested that one or more lipoid substances may be released from the endometrium during menstruation, and may stimulate the contractions of the myometrium and possibly plain muscle elsewhere.

We wish to thank Professor C. S. Russell and other members of the medical and nursing staff of the Jessop Hospital for Women, for providing us with uterine tissues from patients; Dr Marthe Vogt, F.R.S., for her helpful comments; Sandoz Products Ltd. and Bayer Products Ltd. for gifts of BOL and noradrenaline bitartrate; the donors of menstrual material; Miss P. Hopkinson for her able technical assistance, and Mr K. Curtis for drawing Fig. 1. The work was supported by grants from the Medical Research Fund of Sheffield University.

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THE SIMULTANEOUS OCCURRENCE OF CERTAIN CHANGES IN UTERINE AND RENAL ACTIVITY IN DOGS, AND THE ROLE OF OXYTOCIN IN THESE PHENOMENA

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In a previous communication Abrahams & Pickford (1954) described how in dogs the spontaneous uterine contractions which appeared following ovariectomy could be increased in size and/or frequency not only by the intravenous injection of oxytocin, but also by the intravascular (intravenous or intracarotid) injection of hypertonic solutions of NaCl or sucrose. The hypertonic solutions simultaneously induced antidiuresis. On the other hand, uterine contractions ceased abruptly or became minimal during the polyuric phases of diabetes insipidus, though they were large during the normal interphase. It was suggested that the link between the uterus and kidney lay in the posterior lobe of the pituitary, that oxytocin might be necessary for the maintenance, and perhaps also for the appearance of uterine contractions, and that the simultaneity of the uterine and renal effects in normal animals depended on the fact that stimulation always liberated both oxytocin and vasopressin from the supraoptico-hypophysial system (Harris, 1955). Clearly, further examination of these hypotheses was desirable. If oxytocin was necessary for the appearance of spontaneous uterine contractions, then no activity should appear if diabetes insipidus was induced before removal of the ovaries. A further test was perhaps possible, based on the observation of Vogt (1953) and confirmed by van Dyke, Adamsons & Engel (1955), that in the dog oxytocic activity is minimal in hypothalamic tissue. Thus, there was a chance that removal of the posterior lobe alone would leave enough available vasopressin to maintain a normal water balance, but would so reduce circulating oxytocin that uterine contractions would either cease, or fail to appear. By using an osmotic stimulus and observing renal function in some detail it might be possible to determine whether, after removal of the posterior lobe, vasopressin, or both vasopressin and oxytocin were liberated. Since amongst other things excretion of Na was examined the osmotic stimulus used was the intravenous injection of hypertonic sucrose solution (Verney, 1947). The results of such experiments are described below and do, in part, support the original hypotheses.

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METHODS

Five bitches all weighing between 15 and 16 kg were used. Uterine fistulae were prepared and uterine activity recorded as described by Abrahams & Pickford (1954, 1958). The ovaries were removed through a mid-line abdominal incision and operations on the pituitary and hypothalamus were performed transorally. The daily fluid intake of the dogs was recorded before and after pituitary operations. Renal function tests involved observations on the excretion of water, Na, K and Cl. All dogs were hydrated with 250 ml. water at 12 noon and experimental observations were begun between 2 and 2.30 p.m. of the same day, when either the dogs received no more water and urine was collected at a resting rate of flow, or they were given 20 ml./kg water and diuresis was induced. Urine was collected through a short self-retaining intravesical catheter. Na and K were estimated by flame photometry, and Cl by the method of Prout-Winter (Cole, 1919). The vasopressin used was the highly purified natural arginine vasopressin supplied by Dr du Vigneaud and Dr H. B. van Dyke. The oxytocin was either the natural highly purified product, given by Dr du Vigneaud, or the synthetic oxytocin (Syntocinon) made by Messrs Sandoz. The doses of these substances used were made up in 1 ml. 0·9% (w/v) NaCl solution. The sucrose solution used was always 0·89 M.

After death, in two of the four dogs on which hypothalamic operations were performed, serial sections of the pituitary and hypothalamus were examined, every fifth section of 8μ thickness being stained with toluidine blue or by Gomori's method (1941). In two dogs whose posterior lobes were removed the oxytocic and vasopressor activities of the hypothalamus were assayed by Dr M. Holzbauer of the Department of Pharmacology, University of Edinburgh.

RESULTS

Ovariectomized dogs

Some earlier observations (Abrahams & Pickford, 1954; Brooks & Pickford, 1957) on the effect of vasopressin, oxytocin and hypertonic solutions on the kidney and uterus were repeated and amplified, and it was ascertained that the four dogs later subjected to operations on the hypothalamus and pituitary all showed similar, and therefore presumably normal, responses. A fifth dog was kept under observation for a considerable time after ovariectomy to determine how long uterine activity persisted. The uterus of this dog (Jill) ceased activity 70 days after ovariectomy, confirming the observation of Abrahams & Pickford (1954) that activity persisted for at least 43–50 days. The responses of Jill were typical of the group and are shown in Figs. 1–3. In Fig. 1*A* it can be seen that during water diuresis the intravenous injection of hypertonic sucrose solution caused antidiuresis, enhanced the rate of excretion of Na, K and Cl, and increased uterine activity; oxytocin (Fig. 1*B*) in the doses used caused a short-lasting antidiuresis, had little effect on the excretion of electrolytes and increased uterine activity; vasopressin (Fig. 1*C*) was strongly antidiuretic, caused a marked increase in Na, K and Cl excretion, but did not stimulate the uterus. Fig. 2*A* shows that in the non-diuretic state sucrose solution again raised Na, K and Cl loss and increased uterine activity; oxytocin (Fig. 2*B*) raised the rate of excretion of electrolytes and enhanced uterine activity; vasopressin (Fig. 2*C*) had no effect on uterine activity, but on this occasion somewhat increased the rate of electrolyte excretion. This latter action of vasopressin is not invariable at a low rate of urine flow (Anslow & Wesson, 1955; Brooks & Pickford, 1957). It has been suggested that an osmotic stimulus liberates both vasopressin and oxytocin (Abrahams & Pickford, 1954) from the supraoptico-hypophysial system. As may be seen in Fig. 3*A* (non-diuretic state) and Fig. 3*B* (diuretic), the response to a mixture of vasopressin and oxytocin is not unlike that to hypertonic sucrose solution. Throughout, and in all dogs, the renal and uterine responses to the injections began at the same time.

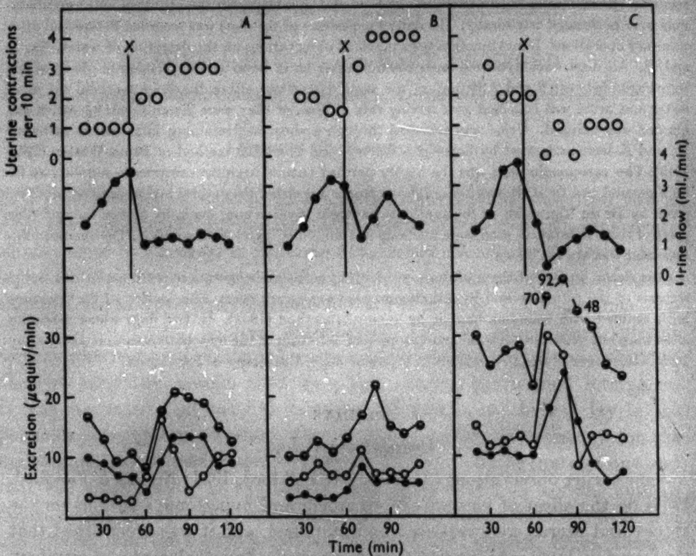

Fig. 1. Uterine contractions and water, Na, K and Cl excretion during water diuresis (normal dog). In each case, starting from the top, number of uterine contractions per 10 min, rate of urine flow, and rate of excretion of Na (●), K (○) and Cl (◒). Intravenous injections made at *X*; in *A*, 15 ml. 0·89 M sucrose solution; in *B*, 100 m-u. oxytocin; in *C*, 2·5 m-u. vasopressin.

Effect of ovariectomy after induction of diabetes insipidus

These observations were made on one dog (Meg) in which diabetes insipidus had been induced by transection of the supraoptico-hypophysial tracts. That the operation was successful was shown during life by the polyuria (Table 1).

After death the histological findings showed that on one side all supraoptic cells had disappeared, and on the other a few rostral, and still fewer, caudal cells were present. In a considerable proportion of these cells the Nissl granules lay close to the nuclei. Paraventricular cells were greatly reduced in number. Twenty-one days after the operation, when the animal had settled into the permanent phase of the diabetes, both ovaries were removed. From the time of the first (hypothalamic) operation daily recordings were made from the uterus. Up to 10 days following ovariectomy the uterus showed no sign of activity. For the following 4 days the dog was given subcutaneously a total of 40 u. oxytocin divided into eight equal doses. Observations on the uterus were made for a further period of 2½ weeks, but at no time was any activity seen. During this period neither oxytocin nor sucrose solution caused any increase in the urinary loss of Na or K, nor did they induce any contraction of the uterus (Fig. 4*A*, *B*). Some discussion has been published elsewhere (Brooks & Pickford, 1958) of the loss of renal response to oxytocin in dogs with diabetes insipidus.

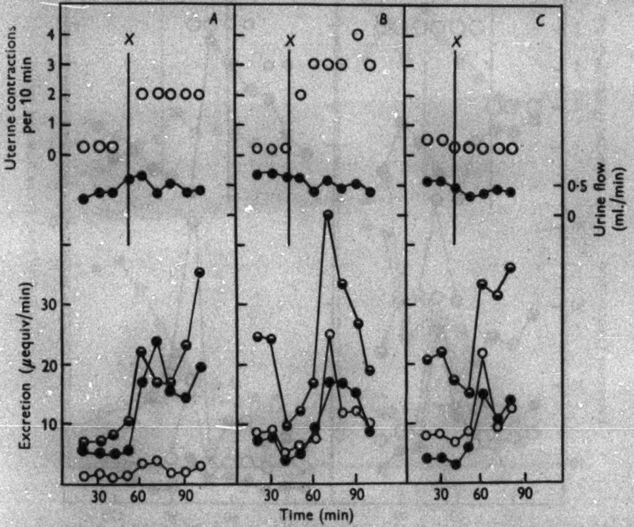

Fig. 2. Uterine contractions and water, Na, K and Cl excretion in the non-diuretic state (normal dog). In each case, starting from the top, number of uterine contractions per 10 min, rate of urine flow, and rate of excretion of Na (●), K (○) and Cl (◒). Intravenous injections made at *X*; in *A*, 15 ml. 0·89 M sucrose solution; in *B*, 100 m-u. oxytocin; in *C*, 3 m-u. vasopressin.

Effect of ovariectomy after posterior lobe removal

In one dog (Sophy) the posterior lobe was removed, and 6 days later when the phase of maximal polyuria was at an end (Table 1) ovariectomy was performed. In this animal only uterine activity and water excretion were examined, since any injection caused strong emotional reactions. Before and between the operations the uterus showed very small occasional contractions of a type sometimes seen in anoestrous animals (Fig. 5*A*) (Abrahams & Pickford, 1954). No observations could be made until the 6th day after ovariectomy because the abdominal wound broke down with extensive herniation of abdominal contents. However, by the 6th day recovery was excellent, and from then until the 9th day after ovariectomy uterine contractions were

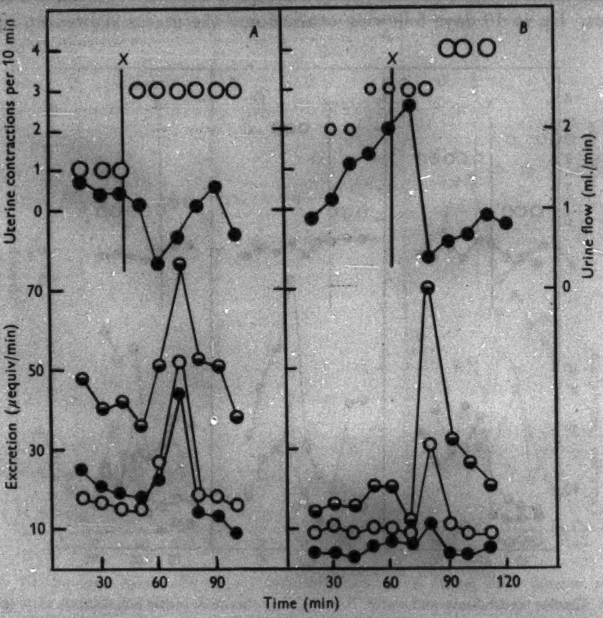

Fig. 3. Uterine contractions and water, Na, K and Cl excretion before and after the intravenous injection at *X* of 100 m-u. oxytocin + 2·5 m-u. vasopressin (normal dog). In both cases, starting from the top, number of uterine contractions per 10 min, rate of urine flow, and rate of excretion of Na (●), K (○) and Cl (◒). *A*, non-diuretic state; *B*, during water diuresis. In *B* uterine contractions increased in size by about four times, as well as in frequency: the former has been indicated by the size of the circles.

regular and of moderate size (Fig. 5*B*). Thereafter contractions became small, infrequent and irregular (Fig. 5*C*). From the 14th day after ovariectomy, which was the 21st day after posterior lobe removal, there were either no contractions at all, or a few very small ones similar to the anoestrous type seen before ovariectomy. On the 24th day after ovariectomy 0·5 mg stilboestrol diproprionate in oil was injected subcutaneously. On the day immediately following, the contractions seen were larger than at any other time. Regular observations were continued for a further 11 days, during which uterine activity was either absent or anoestrous in type, except that on the 8th and 11th days after stilboestrol (Fig. 5*D*) moderate sized contractions occurred once in every 10–13 min. Thirty days after stilboestrol administration and 54 days after ovariectomy a final observation was made, and no hint of uterine activity seen (Fig. 5*E*).

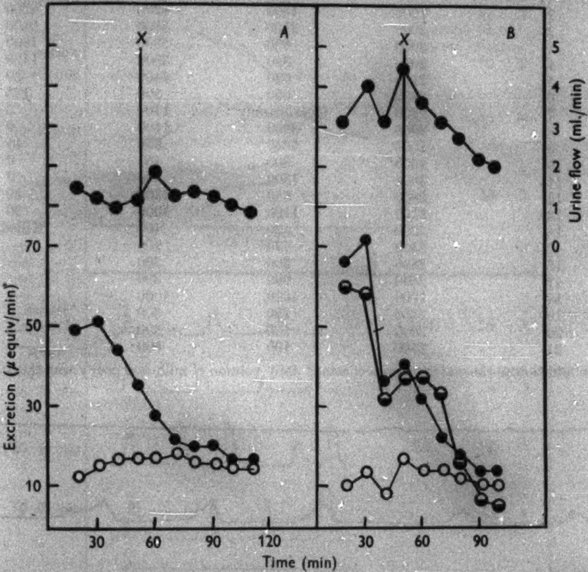

Fig. 4. Excretion of water, Na, K and Cl in dog (Meg) with diabetes insipidus before and after intravenous injection at *X* of 20 ml. 0·89 M sucrose solution. In both cases, starting from the top, rate of urine flow, and rate of excretion of Na (●), K (○) and Cl (◒). *A*, non-diuretic state; *B* during diuresis. The uterus showed neither spontaneous nor induced activity.

After death, histological examination showed that the posterior lobe had been removed and that the anterior lobe appeared in good condition. There

Table 1. Twenty-four hour water intake (ml.)

	Meg	Sophy	Jess	Little Klaus
Condition of dog ...	Diabetes insipidus	Normal	Ovariectomized	Ovariectomized
Range of water intake before operation ...	1400–3000	100–170	120–200	50–100
Operation performed	Ovariectomy	Removal posterior pituitary	Removal posterior pituitary	Removal posterior pituitary
Days after operation				
1	1700	4500	4200	4250
2	2000	2720	5800	6100
3	3000	600	5300	1800
4	2040	380	3900	1120
5	2100	620	2400	20
6	2300	690	200	125
7	2800	Ovariectomy	1100	0
8	2000	3600	1100	0
9	2600	5640	600	45
10	2500	800	400	0
11	3000	1300	400	0
12	2800	920	1300	20
13	2100	1100	1000	20
14	2200	520	400	Killed
15	2300	770	900	—
16	2800	200	700	—
17	2500	300	800	—
18	1900	1000	1000	—
19	2400	280	600	—
20	2600	100	800	—
21	2500	100	600	—

In addition to the water taken a constant daily volume of milk and gravy was taken.

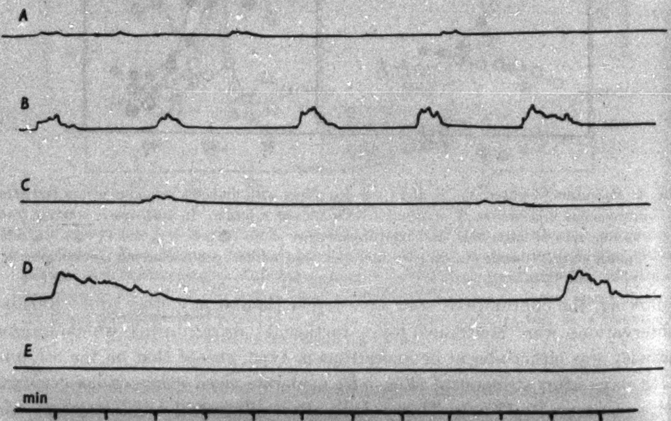

Fig. 5. Tracings from records of spontaneous uterine contractions (Sophy). *A*, 5 days after posterior lobe removal; *B*, 14 days after posterior lobectomy and 8 days after ovariectomy (these were the largest and most frequent contractions seen); *C*, 28 days after posterior lobectomy and 20 days after ovariectomy; *D*, 45 days after posterior lobectomy and 11 days after 0·5 mg stilboestrol; *E*, 54 days after ovariectomy.

was a marked reduction in the number of supraoptic and paraventricular cells, and a large proportion of the remaining cells were ghost-like with fine diffuse granulations; the paraventricular cells in particular appeared swollen. The Gomori staining was only of moderate intensity.

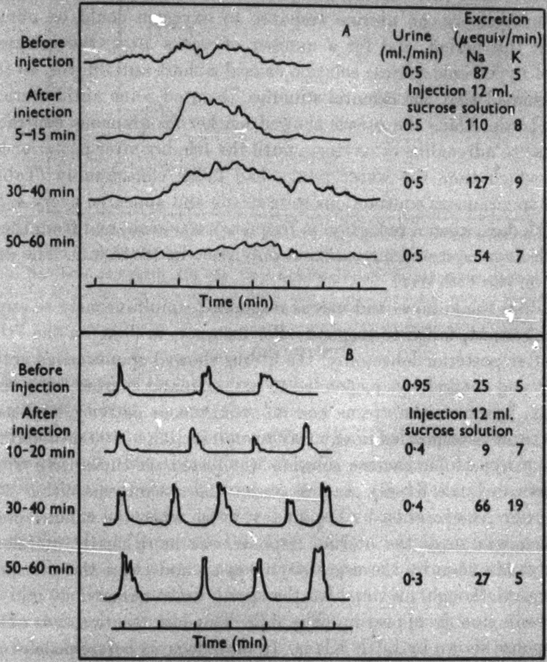

Fig. 6. **Effect of intravenous injection of 12 ml. 0·89 M sucrose solution on uterine activity and on water, Na and K excretion in non-diuretic state (Jess). *A*, ovariectomized; *B*, ovariectomized and posterior lobe removed. In both cases, on left, time of observation; in centre tracings of typical uterine contractions; on right, rate of urine flow and of excretion of Na and K.**

Effect of ovariectomy before removal of the posterior lobe

In two dogs, Jess and Little Klaus, subjected to ovariectomy spontaneous uterine contractions appeared in 6 and 10 days, respectively. Both animals showed normal responses to intravenous injections of hypertonic sucrose solution and oxytocin (Fig. 6*A* and as in Figs. 1, 2). The posterior lobe of the pituitary was removed 11 days after ovariectomy in Jess, and 13 days after in Little Klaus. The uterus of the former showed no spontaneous activity on the 1st day after posterior lobectomy, but some activity was present on the 2nd day and was well developed by the 3rd, at a time when water intake was still high (Table 1). Observations were made on this dog nearly every day for the next 4 weeks, and it was found that between the 18th and 21st days after posterior lobectomy (i.e. within 34 days of ovariectomy) all uterine activity ceased. Furthermore, no uterine response to oxytocin could be obtained. Throughout this period, and for a number of weeks after, the intravenous injection of hypertonic sucrose solution caused a short antidiuresis, so that it may be assumed that the osmotic stimulus liberated some antidiuretic hormone. In Little Klaus the uterus showed neither spontaneous contractions nor response to adrenaline or oxytocin until the 4th day after posterior lobectomy, by which time the water intake had fallen considerably (Table 1). Thereafter spontaneous contractions were strong and appeared every 4–5 min until the 9th day, when a reduction in frequency was seen, and from the 10th to the 13th days contractions occurred only once in 12–13 min. The animal was killed on the 13th day.

In both dogs the kidneys and uterus responded simultaneously to stimulation, or, in concert, failed to respond. For instance, in Jess, on the 7th and 9th days after posterior lobectomy, the uterus showed spontaneous activity, and both it and the kidneys responded to oxytocin and sucrose solution. On the 8th day, however, the uterus had no spontaneous activity, and neither uterus nor kidneys responded in any way to an injection of 100 m-u. oxytocin. The effect of hypertonic sucrose solution was tested six times (five times in Jess and once in Little Klaus). In Jess once it did not increase either uterine contractions or Na excretion by the kidney, twice when the enhancement of Na excretion was small the uterine response was more nearly matched by 30 m-u. oxytocin than by the usual 80–100 m-u., and twice the uterine and renal responses though maximal at the usual time, namely, 40 min after injection, were slow in appearing (Fig. 6*B*). This last reaction was also the type of response shown by Little Klaus. In both dogs, as before posterior lobe removal, vasopressin had no demonstrable effect on the uterus and exerted an immediate antidiuretic action. Oxytocin had its normal immediate effect on the uterus and on the excretion of Na by the kidney (Fig. 7).

Post-mortem assay of hypothalamic tissue from Jess was not wholly satisfactory, but it appeared that the ratio of vasopressin: oxytocin was approximately normal. Assay of hypothalamic tissue from Little Klaus was satisfactory and showed oxytocic activity equivalent to 150 m-u. and vasopressor activity equivalent to 1750 m-u. These values are similar to those found by van Dyke, Adamsons & Engel (1955) in normal dogs. Anterior lobe and stalk activity was equivalent to 6 m-u. oxytocin and 13 m-u. vasopressin respectively.

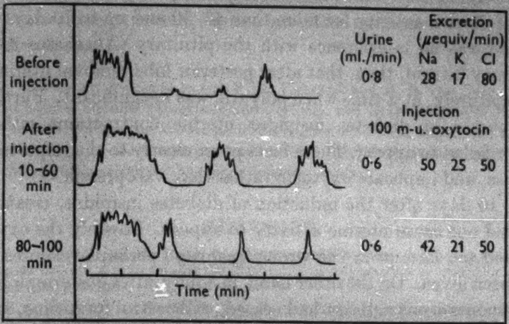

Fig. 7. **Effect of intravenous injection of 100 m-u. oxytocin on uterine activity and on water, Na, K and Cl excretion in dog (Little Klaus) from which both the ovaries and the posterior lobe had been removed. On left, time of observation; in centre, tracings showing typical pattern of uterine contractions; on right, rate of urine flow and rate of excretion of Na, K and Cl.**

DISCUSSION

The results described show that in normal dogs, providing the uterus contracts spontaneously, both it and the kidneys are simultaneously activated by hypertonic sucrose solution, by oxytocin and by mixtures of oxytocin and vasopressin, but that the kidney alone is stimulated by vasopressin. The assumption made is that the sucrose solution affects the uterus by virtue of the oxytocin it liberates, and the kidney by virtue of the vasopressin and oxytocin liberated (Brooks & Pickford, 1957). That any peripheral action of sucrose solution is of minor importance is shown by the fact that in diabetes insipidus it activates neither uterus nor kidneys. A study of the figures shows that the changes in uterine movements cannot be directly or indirectly related to the rate of electrolyte excretion.

The one instance in which there was a lack of complete parallelism between uterus and kidneys was in Jess, in whom spontaneous contractions appeared at a time when there was still a fair degree of polyuria. Apart from that, in the three dogs from which the posterior lobe alone was removed, as long as the uterus showed spontaneous activity, it and the kidneys responded simultaneously to stimulation, even when the timing of their responses was abnormal. This is strong evidence that the changes in both renal and uterine activity were dependent on loss of pars nervosa tissue. An interesting result of posterior lobe removal was that in the two dogs observed for a considerable length of time cessation of uterine activity was related to the date of loss of the posterior lobe, and not to the time of ovariectomy. In both animals the spontaneous contractions disappeared 18–21 days after posterior lobe removal, or 14 and approximately 34 days after ovariectomy. This may be compared with the duration of uterine activity for more than 43, 50 and up to 70 days in dogs in which there was no interference with the pituitary (Abrahams & Pickford, 1954). It may be said, then, that after posterior lobe removal uterine activity failed prematurely, at a time when polyuria was insignificant. Further, in the polyuric phases of diabetes insipidus uterine contractions either ceased abruptly or failed to appear. These facts point clearly to the importance of the pars nervosa, and implicate oxytocin rather than vasopressin.

In Meg, 10 days after the induction of diabetes insipidus, treatment with oxytocin did not cause uterine activity to appear. Possibly the oxytocin was administered too late, or in the wrong quantity; perhaps vasopressin should also have been given. On the other hand, in Sophy, after posterior lobe removal and when spontaneous activity had ceased, stilboestrol for a time, and irregularly, caused contractions to reappear. The stilboestrol may have sensitized the uterus or some tissue controlling uterine activity, to oxytocin still present, though in low concentration, or it may have had a wholly different undetermined action. It is, however, not surprising that the appearance of uterine activity should depend on more than one factor.

Since an isolated uterus in an organ bath shows rhythmical activity it is clear that, for a time at least, movement can occur in the absence of any circulating hormone, including oxytocin. Nevertheless, in the ovariectomized animal activity did not develop in the absence of oxytocin and vasopressin (diabetes insipidus) and ceased prematurely when oxytocin was not readily available (after removal of the posterior lobe). This suggests that in addition to its direct augmentor effect on uterine contractions oxytocin has some other, and undetermined, action on which spontaneous activity indirectly depends.

SUMMARY

1. Observations were made on uterine activity and renal function in ovariectomized dogs. Spontaneous uterine activity continued up to 70 days after ovariectomy.

2. The intravenous injection of hypertonic sucrose solution caused an increase in uterine activity and in Na and Cl excretion by the kidney, the two responses showing a close correlation in time.

3. No spontaneous uterine contractions developed in a dog in which diabetes insipidus was induced before ovariectomy. The kidneys of this dog showed no increase in Na excretion on injection of sucrose solution, nor could any response be induced in the uterus.

4. In dogs from which the posterior lobe of the pituitary had been removed, uterine activity ceased prematurely at a date related to the removal of the posterior lobe and not to the ovariectomy.

5. It is concluded that the hormones of the pars nervosa, and oxytocin in particular, have some importance in the onset and maintenance of spontaneous uterine contractions.

We wish to express our gratitude to Drs V. du Vigneaud and H. B. van Dyke who gave us highly purified natural arginine vasopressin and oxytocin, and to Dr Holgate of Messrs Sandoz who supplied us with Syntocinon. We are particularly grateful to Dr M. Holzbauer, of the Department of Pharmacology, for the trouble she took in developing an accurate method for assaying vasopressor and oxytocic activity in the hypothalamic tissues.

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THE EFFECTS OF ADRENALINE, NORADRENALINE AND ISOPRENALINE ON SKELETAL MUSCLE CONTRACTIONS IN THE CAT

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Adrenaline, noradrenaline and other sympathomimetic amines have been shown to increase the maximal twitch tension of unfatigued skeletal muscles (Oliver & Schaefer, 1895; Gruber, 1922*a*, *b*; Goffart & Brown, 1947; West & Zaimis, 1949; Brown, Goffart & Vianna Dias, 1950; Goffart & Ritchie, 1952; Huidobro, Cubillos & Eyzaguirre, 1952; Goffart, 1952, 1954; Montagu, 1955). This response, however, is not characteristic of all skeletal muscles. In fact the present experiments show that, in the cat, slow contracting muscles such as the soleus respond in a completely opposite way. A short account of the results has already been published (Bowman & Zaimis, 1955).

METHODS

Cats were anaesthetized either with chloralose only (80 mg/kg) or with a mixture of chloralose (80 mg/kg) and pentobarbitone sodium (6 mg/kg), injected into a subcutaneous vein of the fore-limb or into the saphenous vein of the hind limb.

Recording of muscle contractions

The tibialis anterior and soleus muscles were prepared for recording and the hind limb was pinned and fixed horizontally to a Brown-Schuster myograph stand. When indirect stimulation of the muscles was required, shielded silver electrodes were placed on the sciatic nerve and the nerve was ligated central to the electrodes. Twitches and tetani were excited by square wave pulses of 0·2 msec duration and twice the strength required to evoke a maximal twitch. In experiments in which curarized muscles were directly excited, stimuli of maximal strength and 0·5 msec duration were applied between the tendon of the muscle and the drill in the femur. Electrical contact with the tendon was made through silver wire attached to a saline-soaked pad of cotton-wool. In order to ensure that the current supplied for direct stimulation was not short-circuited through the other tissues, the muscles were insulated by enclosing their bellies in a rubber fingerstall smeared with liquid paraffin. Muscle tension was recorded either on the smoked drum by means of an isometric steel-spring myograph or electrically using a capacitance strain gauge.

Recording of the venous outflow

An incision was made in the skin on the medial surface of the thigh to expose the femoral artery and vein, and, with the exception of the artery chosen for the intra-arterial administration of drugs, all branches of the femoral artery and vein down to the popliteal space were ligated and cut. This involved the removal of part of the gracilis muscle in order to tie off a few small branches of the artery and vein which supply this muscle.

An incision was then made in the skin along the mid line of the back of the leg to expose the popliteal space and the gastrocnemius muscle, and a further incision was made along the medial side of the leg to expose the tibialis posterior artery and vein lying between the soleus and flexor longus hallucis muscles.

Soleus muscle. For the recording of the venous outflow from the soleus muscle the popliteal artery and vein were tied below the tibialis posterior artery and vein. All branches arising from the two last-named, except those supplying the soleus muscle, were then ligated. The popliteal artery and vein pass between the two heads of the gastrocnemius muscle, near their origin, and at this point a small artery and vein arise carrying blood to and from this muscle. In order to overcome the difficulty of reaching these vessels, the medial head of the gastrocnemius was twice ligated near its origin, the muscle cut between the two ligatures and the exposed vessels tied. Finally, the remaining vessels supplying the muscles surrounding the popliteal space were ligated. Thus the blood flow in the femoral artery and vein was only that of the soleus muscle.

Tibialis anterior muscle. For the recording of the venous outflow from the tibialis anterior muscle the anterior tibial artery and vein were ligated near the ankle joint, thus cutting off the circulation of the foot. All branches and tributaries of the anterior tibial artery and vein were then tied, except those supplying the tibialis anterior muscle. Finally, all remaining branches of the popliteal artery and vein were ligated.

The skin was then sewn back into position and the leg mounted horizontally on a Brown-Schuster myograph stand. For the recording of the blood flow, the flowmeter described by Hilton (1952, 1953) was used. Ten minutes before the cannulation of the vein, heparin (1000 u./kg) was administered intravenously. At the end of each experiment indian ink was injected through the arterial cannula in order to ascertain that the blood flow was in fact only that of the muscle under study.

Administration of drugs

Drugs were injected intravenously or intra-arterially. For the intra-arterial administration a cannula made from a hypodermic needle was tied into the cut central end of a branch of the femoral artery, usually the small artery supplying the gracilis muscle. A micro-syringe was used for the administration of the drugs. Thus a minute volume could be injected straight into the blood stream of the femoral artery; 0·01 ml. was the maximum volume of any solution administered. The same volume of 0·9% saline was used as a control.

Stimulation of splanchnic nerves and of the lumbar sympathetic chain

When the effects of splanchnic stimulation were studied, the left splanchnic nerve was prepared and stimulated by means of silver hook electrodes. In experiments in which the effects of stimulation of the lumbar sympathetic chain were studied, muscle contractions were elicited by stimulation of the motor roots. The preparation was that described by Bülbring & Burn (1939).

Drugs

The sympathomimetic amines used were L-adrenaline bitartrate, L-noradrenaline bitartrate and isoprenaline sulphate. The doses quoted in the text refer to the quantity of amine calculated as base. All solutions were made in 0·9% (w/v) NaCl saline.

RESULTS

Twitch

All three amines, adrenaline, noradrenaline and isoprenaline, administered intravenously or intra-arterially increased the twitch tension of the tibialis anterior and decreased that of the soleus muscle.

Fig. 1 illustrates an experiment in which the maximum twitch tension of the tibialis anterior and soleus muscles were recorded simultaneously on a smoked drum. 50 μg of adrenaline injected intravenously produced an increase in the twitch tension of the tibialis but a decrease in that of the soleus muscle. Figs. 2 and 3 show the effect of adrenaline on the time course of isometric twitches recorded electrically. In the experiment illustrated by Fig. 2 an intra-arterial injection of 10 μg of adrenaline increased the twitch tension of the tibialis anterior muscle by 13% and altered its time course; the rate of development of tension was slightly diminished, the time to peak tension was increased by 15% and the over-all duration of the twitch by 17%. On the other hand, in the experiment illustrated by Fig. 3 an intravenous injection of 5 μg/kg of adrenaline not only decreased the twitch tension of the soleus muscle by 18%, but markedly altered the time course of the contraction. The

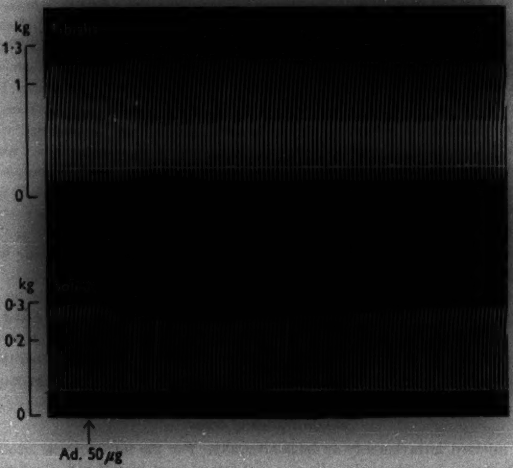

Fig. 1. Cat, 2·8 kg. Simultaneous recording of indirectly excited maximal twitches of the tibialis anterior and soleus muscles once every 10 sec. At arrow, 50 μg adrenaline administered intravenously.

time to peak tension was reduced by 25%, the duration of the twitch by 33% and the rate of relaxation was markedly increased. Results such as those illustrated by Figs. 2 and 3 are typical of all three sympathomimetic amines.

Of the three amines, isoprenaline was slightly more potent than adrenaline in producing these effects, and noradrenaline was the least potent. The minimal effective doses are shown in Table 1.

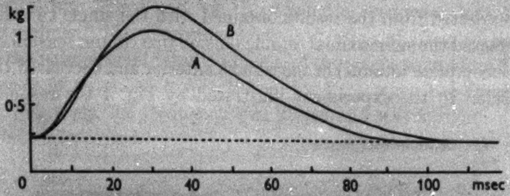

Fig. 2. Maximal twitches of the tibialis anterior muscle elicited by indirect stimulation once every 10 sec and recorded electrically, *A*, before adrenaline and *B*, 120 sec after the intra-arterial injection of 10 μg adrenaline.

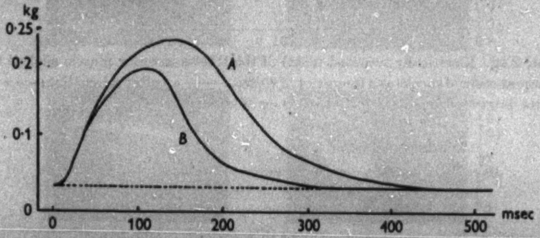

Fig. 3. Cat, 3·1 kg. Maximal twitches of the soleus muscle elicited by indirect stimulation once every 10 sec and recorded electrically, *A*, before adrenaline and *B*, 70 sec after the intravenous administration of 15 μg adrenaline.

TABLE 1

	Minimal effective intravenous dose (μg/kg) of		
Muscle	Adrenaline	Noradrenaline	Isoprenaline
Tibialis anterior	3–10	5–15	2–8
Soleus	0·06–0·5	5–20	0·06–0·4

Although the sensitivity of the soleus to adrenaline and isoprenaline is much greater than that of the tibialis anterior muscle, there is little difference between the doses of noradrenaline required to affect them. This makes the soleus muscle approximately 50 times less sensitive to noradrenaline than to adrenaline and isoprenaline.

The time course of the effects of all three amines is similar in both muscles. The effect usually starts 20–40 sec after the intravenous or intra-arterial administration of the drug and reaches its peak 40–60 sec later. The duration of the effect depends on the magnitude of the dose and may last 12–15 min after amounts producing maximum responses have been administered. 16% was the maximum increase in twitch tension recorded from the tibialis anterior and 20% the maximum decrease from the soleus muscle.

Tetanus

As was expected from the results obtained with the single twitch all three amines increased the submaximal tetanic tension (maximum tension developed during an incomplete tetanus) of the tibialis anterior and decreased that of the soleus muscle. In the experiment illustrated by Fig. 4 the response of the tibialis anterior muscle to a stimulation frequency of 20/sec was electrically recorded before and after adrenaline. After adrenaline the tension developed was greater and fusion was facilitated. Fig. 5 illustrates the results of a similar experiment on the soleus muscle. After adrenaline the tension developed was less and the degree of fusion markedly decreased. Fig. 6 illustrates similar effects on the soleus muscle with isoprenaline. Tetani of 3 sec duration and 14/sec frequency were recorded every 30 sec on a rapidly revolving smoked

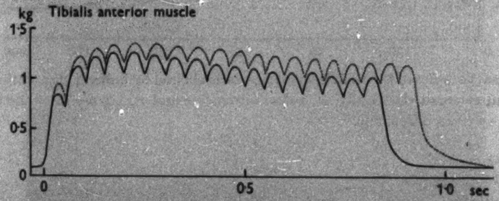

Fig. 4. Cat, 2 kg. Electrically recorded tetani of the tibialis anterior muscle elicited indirectly with supramaximal shocks at a frequency of 20/sec. ———, before adrenaline; – – – –, 140 sec after the intravenous administration of 30 μg of adrenaline.

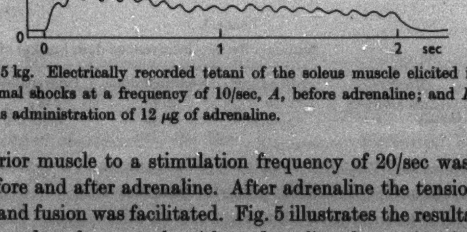

Fig. 5. Cat, 2·5 kg. Electrically recorded tetani of the soleus muscle elicited indirectly with supramaximal shocks at a frequency of 10/sec, *A*, before adrenaline; and *B*, 140 sec after intravenous administration of 12 μg of adrenaline.

drum. After 5 μg/kg of isoprenaline had been administered intravenously, the tension and the degree of fusion progressively decreased so that 90 sec later the muscle relaxed almost completely between stimuli.

The lower the frequency of stimulation the more marked the effects obtained. For example, in one experiment 30 μg of adrenaline, administered intravenously, increased the submaximal tetanic tension of the tibialis anterior muscle by 11·5% when the frequency of stimulation was 14/sec, by 3·6% when the frequency was 20/sec and by 1% when the frequency was 50/sec. In another experiment, the intravenous administration of 15 μg of isoprenaline decreased the submaximal tetanic tension of the soleus muscle by 36% when the frequency of stimulation was 10/sec, by 18% when the frequency was 14/sec, by 7% when the frequency was 23/sec and was without effect when the frequency was 30/sec.

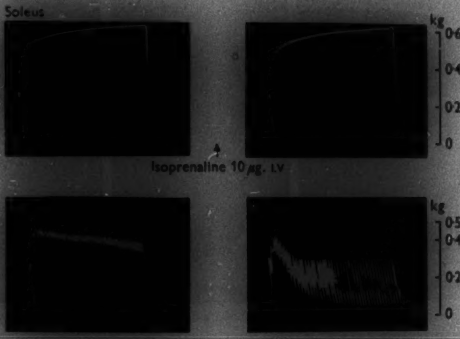

Fig. 6. Cat, 2·2 kg. Electrically recorded tetani of the soleus muscle elicited indirectly every 30 sec by stimulation with supramaximal shocks at a frequency of 14/sec. 1, before isoprenaline; 2, 3 and 4, after the intravenous administration of 10 μg of isoprenaline.

The doses of the three sympathomimetic amines needed to affect a tetanus were similar to those needed to affect the single twitch. Adrenaline, noradrenaline and isoprenaline administered intravenously or intra-arterially in doses not causing a marked vasoconstriction were without effect on the maximum tetanic tension of either muscle.

Concomitant blood-flow changes. The majority of workers have concluded that the effects of the sympathomimetic amines on skeletal muscles are independent of blood-flow changes; in most cases, however, the evidence is indirect. In order to obtain more direct information, in the present experiments the action of the sympathomimetic amines on the blood flow through the muscle under study was simultaneously recorded.

Isoprenaline, injected intravenously or intra-arterially, always increased the blood flow through the muscle. On the other hand, whereas an intra-arterial injection of noradrenaline always decreased the blood flow, an intravenous one produced, according to its magnitude and its effect on the general blood pressure, either a decrease or a biphasic response. Finally, adrenaline produced an increase, a decrease or a biphasic response by either route of administration, again according to the dose and the general blood-pressure effect.

Whatever the blood-flow response the effects of the three sympathomimetic amines on the single twitch remained unaltered. Such results are illustrated by Figs. 7 and 8. In another series of experiments, in which the vasoconstrictor action of adrenaline and noradrenaline was abolished by the previous administration of dibenamine or phentolamine, the sympathomimetic amines produced the usual effects on muscle contraction.

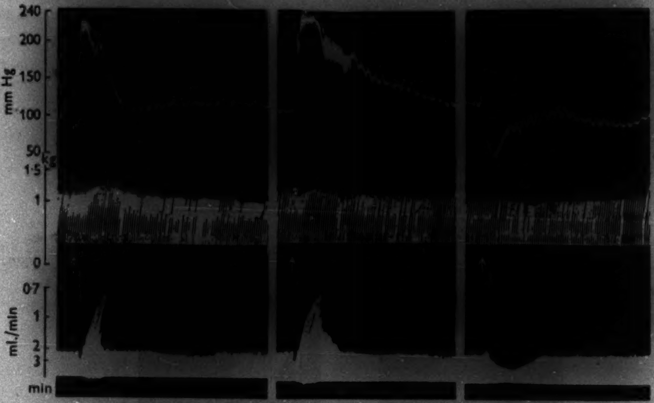

Fig. 7. Blood pressure, indirectly excited maximal twitches of the tibialis anterior muscle and venous outflow from the tibialis anterior muscle recorded simultaneously. At arrow (1), 50 μg of adrenaline; at arrow (2), 50 μg of noradrenaline and at arrow (3), 50 μg of isoprenaline administered intravenously at intervals of 1 hr.

Tetanic contractions, however, may be altered by a powerful vasoconstriction. For example, adrenaline or noradrenaline in large doses may cause depression of the submaximal or maximal tetanic tension of the tibialis anterior muscle. Fig. 9 illustrates this effect. Both vasoconstriction and reduction of tetanic tension were more pronounced after noradrenaline. That this reduction of the tetanic tension is due to a decreased blood flow was further confirmed in experiments in which dibenamine or phentolamine were previously administered. In such experiments when the vasoconstrictor action of adrenaline and noradrenaline was reduced or abolished, the reduction of the tetanic tension did not occur. The maximum tetanic tension of the soleus

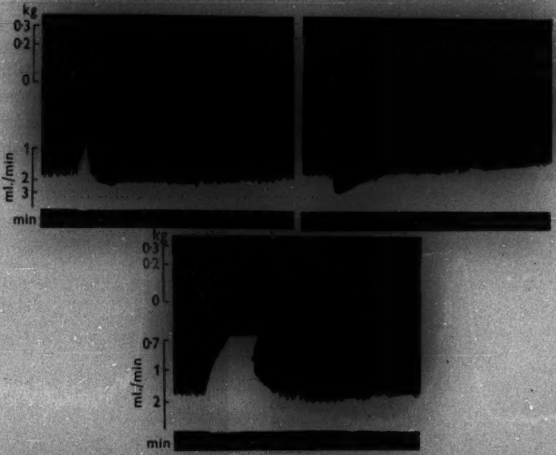

Fig. 8. Cat, 4·5 kg. Indirectly excited maximal twitches and venous outflow from the soleus muscle recorded simultaneously. At arrow (1), 1 μg of adrenaline; at arrow (2), 1 μg of isoprenaline and at arrow (3), 5 μg of noradrenaline administered intra-arterially at intervals of 30 min.

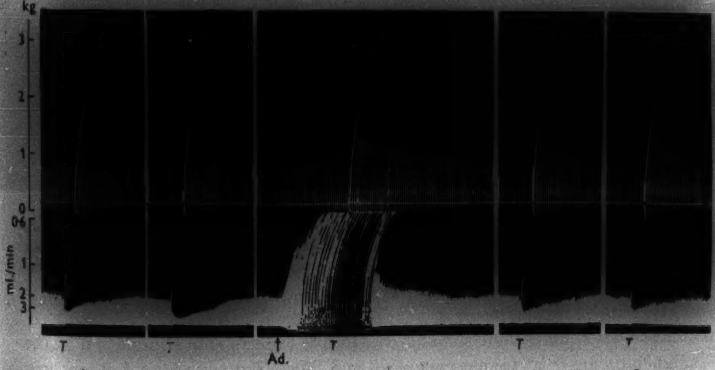

Fig. 9. Cat, 3·3 kg. Maximal twitches elicited by indirect stimulation every 10 sec and venous outflow from the tibialis anterior muscle recorded simultaneously. T = tetanus of 2 sec duration and 120/sec frequency, interpolated every 15 min; at arrow, 10 μg of adrenaline administered intra-arterially.

muscle, however, was never reduced even in the presence of a marked vasoconstriction.

Direct stimulation. In order to study the effects of the three sympathomimetic amines on the twitch and tetanic tensions of the directly stimulated tibialis and soleus muscles, the drugs were administered after neuromuscular transmission was completely blocked by a slow continuous infusion of tubocurarine. In both muscles the effects produced were similar to those obtained when the contractions were elicited by indirect stimulation.

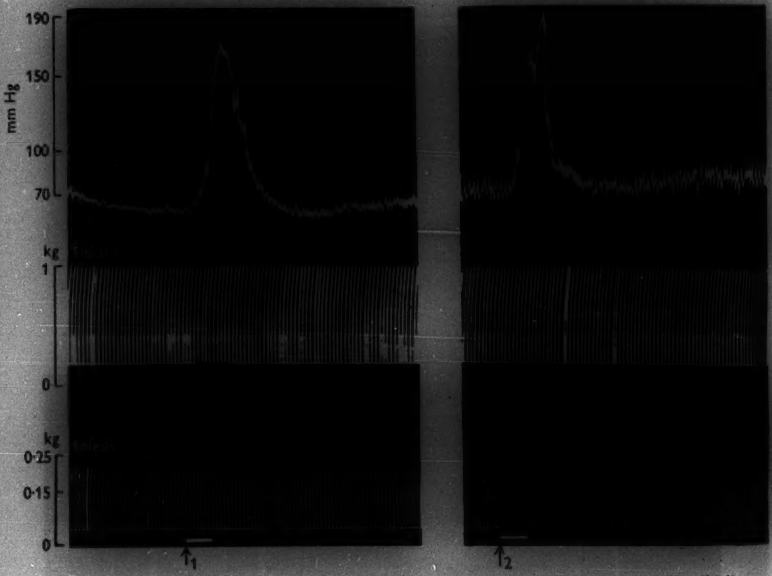

Fig. 10. Cat, 2·3 kg. Blood pressure and indirectly excited maximal twitches of the tibialis anterior and soleus muscles recorded simultaneously. At arrow (1) stimulation of the left splanchnic nerve for 60 sec at a frequency of 20/sec; at arrow (2) 10 μg of adrenaline administered intravenously during 60 sec.

The effect of splanchnic stimulation. In five experiments stimulation of the splanchnic nerve at frequencies of 10 and 20/sec for periods of 1–2 min was accompanied by a decrease of the maximal twitch tension of the soleus muscle. In four out of the five experiments and only with the higher frequency of stimulation the maximal twitch tension of the tibialis anterior muscle was increased. The effects of splanchnic stimulation could be closely imitated by the intravenous administration of 3–5 μg/kg/min of adrenaline (Fig. 10).

The effect of stimulation of the lumbar sympathetic chain. In a further series of five experiments maximal twitches of the tibialis anterior and the soleus muscles were elicited by stimulation of the motor roots. Stimulation of the lumbar sympathetic chain at frequencies of 10 and 20/sec for periods up to 5 min produced no change in the maximal twitch tension of the tibialis anterior muscle. In one animal, however, the maximal twitch tension of the soleus muscle was slightly decreased by stimulation at 20/sec.

DISCUSSION

Oliver & Schaefer (1895) were the first to show that extracts of the adrenal medulla increased skeletal muscle contractions. Since then many workers have obtained similar results with adrenaline (Gruber, 1922*a*, *b*; Goffart & Brown, 1947; West & Zaimis, 1949; Brown *et al.* 1950; Goffart & Ritchie, 1952; Goffart, 1952; Huidobro *et al.* 1952; Montagu, 1955); with noradrenaline (West & Zaimis, 1949; Brown *et al.* 1950; Huidobro *et al.* 1952; Goffart, 1952, 1954); and with isoprenaline (Brown *et al.* 1950; Goffart, 1952; Huidobro *et al.* 1952).

The present experiments on the tibialis anterior muscle confirm these findings and at the same time show that the increase in twitch tension produced by the three sympathomimetic amines is associated with a slight decrease in the rate of development of tension and an increase in the time to peak, the over-all duration of the twitch being prolonged. Goffart & Ritchie (1952) reported similar changes with adrenaline in the time course of the maximal twitch of the isolated diaphragm of the rat.

As was expected from the results on the single twitch, all three sympathomimetic amines facilitate fusion and increase the submaximal tetanic tension; the maximal tetanic tension, however, is not increased. When adrenaline and noradrenaline are administered in doses producing a marked vasoconstriction they may depress both submaximal and maximal tetanic tensions. That vasoconstriction is the cause of this depression is supported by the following results: (1) adrenaline and noradrenaline, only in doses which cause an almost complete arrest of the local blood flow, decrease the maximal tetanic tension; (2) noradrenaline is more potent than adrenaline in reducing tetanic tension and at the same time more powerful in decreasing the blood flow through the muscle; (3) isoprenaline, a pure vasodilator, never depresses tetanic tension; and (4) adrenergic blocking substances in doses reducing the vasoconstrictor action of both adrenaline and noradrenaline prevent the depression of tetanic tension. Submaximal tetanic contractions were shown in the present experiments to be more sensitive to vascular changes than maximal tetanic contractions. Similar results were obtained by Dolgin & Lehmann (1930) who showed that in human subjects weak contractions were more sensitive to ischaemia than strong contractions.

Goffart & Ritchie (1952) concluded that adrenaline can depress the maximal tetanic tension of the tibialis anterior muscle. In their experiments, however, they used large doses of adrenaline injected intra-arterially. The present results suggest that the reduction of the maximal tetanic tension they obtained was not due to a direct effect of adrenaline on the muscle contraction but to a simultaneous powerful vasoconstriction.

The results obtained on the soleus muscle show that skeletal muscles vary in their responses to sympathomimetic amines. In contrast to the effects on the tibialis anterior, isoprenaline, adrenaline and noradrenaline decreased the maximal twitch tension of the unfatigued soleus muscle. Electrical records showed that this response is associated with a decrease in the contraction time, a marked increase in the speed of relaxation and a reduction in the over-all duration of the twitch. As a consequence of these effects on the single twitch the degree of tetanic fusion is decreased and the submaximal tetanic tension reduced.

The tibialis anterior muscle is known to consist mainly of white or fast-contracting fibres while the soleus consists mainly of red or slow-contracting fibres. In a few experiments the plantaris and gastrocnemius were used in order to study the effect of the sympathomimetic amines on muscles containing a more even mixture of red and white fibres. The twitch tension of the gastrocnemius muscle was increased by the amines but to a less extent than that of the tibialis anterior muscle, whereas the twitch tension of the plantaris was decreased but to a less extent than that of the soleus muscle. Thus the effect of the amines on the twitch tension appears to depend on the proportion of red and white fibres in the muscle. The contraction of muscles such as the gastrocnemius and plantaris is little affected, possibly because neither type of fibre predominates greatly and the opposite actions on the two types of fibre tend to cancel each other. Goffart (1952), from experiments on muscles containing a mixture of red and white fibres (diaphragm of rat and tenuissimus muscle of cat), concluded that the effect of adrenaline must be the same on both red and white fibres, and he postulated that the postural tone of the soleus muscle would be improved. The results obtained in the present experiments, performed directly on the soleus muscle, give no support to such conclusions.

When considering the possible significance of these results, it is important first of all to find out whether physiological concentrations of sympathin are large enough to affect muscle tension. Several workers have attempted to determine the upper limit of the rate of impulse discharge in the sympathetic nervous system. Bronk, Ferguson, Margaria & Solandt (1936) recorded action potentials from individual sympathetic fibres and concluded that the discharge rate of sympathetic nerve cells never exceeds, even in stress, 10–20 impulses/sec. Others (Rosenblueth, 1932, 1950; Bacq, 1935) determined the frequency of nerve stimulation to which the corresponding effector cells give a maximum response. In mammals this was found to be between 10 and 25 impulses/sec. Folkow (1952*a*, *b*) recorded the blood flow from a limb and matched the vasoconstriction produced by strong reflex excitation of the vasomotor centre with the effect produced by stimulation of the vasoconstrictor nerves themselves at various frequencies. He concluded that the normal peripheral resistance of the small vessels is maintained by quite a low rate of impulse discharge, probably around 1–2 impulses/sec, and that the average rate of impulse discharge accompanying a strong reflex excitation of the vasomotor centre is about 3–5 impulses/sec, the maximum discharge ever obtained being 6–8 impulses/sec. According to Celander (1954) and Folkow (1955) the rate of discharge in sympathetic nerves never exceeds 10 impulses/sec in the intact animal.

In the resting condition the adrenal medullae release approximately 0·1 μg/kg/min of a mixture of adrenaline and noradrenaline (Kaindl & von Euler, 1951; Duner, 1953; Folkow & von Euler, 1954). Celander (1954), assuming that the maximum rate of discharge in the splanchnic nerves is 10 impulses/sec, has calculated that the absolute maximum physiological output of sympathin from the adrenal glands when all cells are simultaneously activated is 5 μg/kg/min. According to him this amount represents the theoretical upper limit of amine secretion in the cat; it is considerably higher than that found during various types of reflex activation. Strong reflex excitation of the vasomotor centre causes the release of 0·2–0·5 μg/kg/min (Kaindl & von Euler, 1951) and in prolonged asphyxia, which appears to be the most powerful physiological stress, only 1–2 μg/kg/min is discharged (von Euler & Folkow, 1953; Celander, 1954). Intense pain-fibre stimulation in lightly anaesthetized cats causes the release of 0·6–0·7 μg/kg/min (von Euler & Folkow, 1953). From these results, Folkow (1955) has reached the conclusion that a rapid injection of 1 μg of adrenaline or noradrenaline administered intravenously to an average sized cat is, from a physiological point of view, a very large dose, creating a peak concentration which in the intact animal might possibly be reached only in extreme stress.

At first sight an increase in muscle tension appears to be more advantageous than a decrease. It is questionable, however, whether the concentration of circulating amines under any circumstances ever reaches a sufficiently high level to affect muscles like the unfatigued tibialis anterior. The present experiments show that the minimal effective intravenous dose of adrenaline necessary to increase the tension of the tibialis anterior muscle is usually between 3 and 10 μg/kg. Such concentrations are considerably greater than those considered by Folkow to be within physiological limits.

In four out of five experiments stimulation of the left splanchnic nerve caused a slight increase in the maximal twitch tension of the tibialis anterior muscle. These results confirm the finding of Gruber (1922*a*) and Goffart (1952).

In the present experiments, in order to produce this effect, it was necessary to stimulate the nerve at a frequency of 20/sec, stimulation at 10 impulses/sec being without effect in all five experiments. Stimulation of the left splanchnic nerve at a frequency of 20/sec may be assumed to cause the liberation of about the same amount of sympathin as is liberated by stimulation of both left and right splanchnic nerves at a frequency of 10/sec, that is, according to Celander (1954) and Folkow (1955), the maximum possible rate of discharge under physiological conditions. The effects of splanchnic stimulation at this frequency were closely imitated by the intravenous administration of 3–5 μg/kg/min of adrenaline. These quantities are in agreement with those found by Celander (1954). Stimulation of the lumbar sympathetic chain, as Goffart (1952) also showed, was without effect on the twitch tension of the unfatigued tibialis anterior muscle.

Thus from the results described it appears unlikely that the effect of sympathomimetic amines on the contractions of muscles such as the tibialis anterior has any physiological significance. However, if it has it will only be during conditions of extreme stress when the amounts of circulating amines might possibly reach a concentration equivalent to that produced by minimal effective doses of adrenaline.

On the other hand, the soleus muscle is considerably more sensitive to adrenaline than the tibialis anterior muscle. This indicates that if adrenaline has any physiological action in relation to unfatigued muscle contractions it will be the slow-contracting or red fibres which are first affected. The present experiments show that the minimal effective intravenous doses of adrenaline necessary to affect this muscle are of the order of 0·06–0·5 μg/kg, doses producing concentrations which are within the range of the physiological output from the adrenal glands. In all experiments stimulation of the left splanchnic nerve at frequencies of 10/sec caused a marked decrease in the twitch tension; the decrease produced by stimulation at 20/sec approached the maximum effect obtained with injected adrenaline. Furthermore, doses of adrenaline which just affect the general blood pressure frequently decrease the tension of the soleus muscle. It seems probable, therefore, that some physiological significance should be attached to the effect on this muscle.

In only one out of five experiments stimulation of the lumbar sympathetic chain at a frequency of 20 impulses/sec caused a very small decrease in the twitch tension of the soleus muscle. Stimulation at 10 impulses/sec was without effect in all experiments. It can be concluded therefore that the sympathetic nerves do not influence the contractions of this muscle under physiological conditions. Since the transmitter of these adrenergic nerves is noradrenaline (Folkow & Uvnäs, 1948) and since the soleus muscle is relatively insensitive to this amine, this result might be expected. Noradrenaline is about 50 times less active than adrenaline on the soleus muscle. This lack of sensitivity to noradrenaline is not surprising, for if the soleus muscle were sensitive to noradrenaline every increase in the activity of the vasoconstrictor nerves would be accompanied by a decrease in the tension of the muscle.

An explanation must be sought, therefore, as to how the effect on muscles like the soleus is of assistance to the over-all skeletal muscle performance. Human muscles are known to consist of a mixture of slow- and fast-contracting fibres and it is possible that in emergencies a speeding up of the slow-contracting fibres would facilitate muscle work by producing a more uniform contraction.

Goffart (1952) concluded that during emergency states adrenaline and noradrenaline would increase the postural tone, improve the respiration and increase the contractions of other muscles. This was probably because at that time it was not realized that the amounts of amines needed to increase the muscle tension were much larger than those normally found under physiological conditions, and also because it was not known that skeletal muscles differ in their responses to sympathomimetic amines.

Denny-Brown (1929) showed that postural tone is maintained in the soleus muscle by rates of firing from 5 to 25/sec, frequencies well within the range affected by adrenaline. During emergency states the tension of the soleus and similar muscles may be decreased by the circulating adrenaline. This may be the cause of the well known feeling of weakness in the limbs experienced as an effect of fright. Barcroft & Swan (1953), describing the effects of an intravenous infusion of adrenaline in man, report that the subjects experience a feeling of fatigue in the back and in both legs and that a coarse tremor of the extremities is common. The same authors, however, were unable to record such effects with noradrenaline. Among the symptoms in patients with phaeochromocytoma, body tremors are described (Pickering, 1955). Usually in such cases paroxysms are produced by the sudden release into the circulation of relatively large amounts of mixtures of noradrenaline and adrenaline, the former tending to predominate. Litchfield & Peart (1956) describe a rather uncommon case of phaeochromocytoma in which the tumour contained more adrenaline than noradrenaline. This patient exhibited unusually pronounced muscle tremors during the attacks (personal communication by Professor Peart). All these skeletal muscle effects could be explained by an action of adrenaline similar to that described in the present experiments on the soleus muscle.

SUMMARY

1. In the cat, adrenaline, noradrenaline, isoprenaline and splanchnic stimulation increase both the indirectly and the directly excited maximal twitches of the tibialis anterior but decrease those of the soleus muscle. Stimulation of the lumbar sympathetic chain is usually without effect on either muscle.

2. Electrical recordings show that the effects on the tension are accompanied

by changes in the time course of the maximal twitch. In the tibialis anterior muscle the rate of development of tension is slightly diminished, whereas the time to peak tension and the duration of the whole twitch are increased. In the soleus muscle, on the other hand, the time to peak tension and the duration of the twitch are reduced and the rate of relaxation markedly increased.

3. All three sympathomimetic amines facilitate fusion and increase the tension of a submaximal tetanus of the tibialis anterior but decrease the tension and the degree of fusion in the soleus muscle. Maximal tetanic tension of both muscles, however, is not affected.

4. Simultaneous recording of the blood flow through the individual muscles shows that these effects are independent of vascular changes.

5. The soleus muscle is considerably more sensitive to adrenaline and isoprenaline than the tibialis anterior muscle but there is little difference in their sensitivities to noradrenaline.

6. The observations have led to the conclusion that whereas physiological concentrations of adrenaline are unlikely to affect fast-contracting muscles they may well influence the activity of slow-contracting muscles.

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THE IONIC BALANCES AND TRANSFERENCES OF THE SHEEP'S PAROTID GLAND DURING MAXIMAL STIMULATION

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In a previous study (Coats & Wright, 1957), which aimed to establish the relationship between the composition of parotid saliva and the rate of salivary secretion, it was shown that there were two distinct phases of salivary composition following stimulation of the motor nerve. The start-up phase (Phase I), as it was defined, lasted for the first 2 min. It was characterized by a rise, a fall, and a subsequent further rise of sodium concentration and a roughly reciprocal change in the salivary potassium concentration, these changes being relative to the concentrations of the ions prevailing under the immediately preceding conditions of basal (denervated) parotid secretion. The anions bicarbonate, chloride and phosphate also showed concentration changes during Phase I, a fall in phosphate being reciprocally related to a combined increase of chloride and bicarbonate concentrations. Following this initial period it was found that for each ion the composition of the saliva was related to the rate of secretion, and this stage of the secretory process was defined as the equilibrium stage or Phase II.

The finding by Burgen (1956) and Coats & Wright (1957) that immediately following motor nerve stimulation the venous potassium concentration was increased above the arterial, together with Lundberg's (1955) observations on potential changes in the submaxillary gland of the cat, suggested that Phase I of salivary secretion involved changes in the composition of the parotid gland itself. For these reasons it seemed of importance to investigate in detail the electrolyte state of the gland during its changing activity from basal (denervated) secretion through its period of readjustment (Phase I salivary flow) to the equilibrium state (Phase II salivary flow), a study involving simultaneous estimation of the glands' arterial inflow and salivary and venous outflow of electrolytes.

METHODS

The data presented were obtained from seven Merino sheep. Each animal, before use, was kept indoors in a cage for 5–7 days and was given 25 g of sodium bicarbonate dissolved in 1 l. of tap water by intraruminal tube 2 days before the experiment, to avoid the possibility of minor grades of sodium depletion (Denton, 1956; Coats & Wright, 1957). In all cases the last urine specimen before experimental use contained sodium at a concentration of 100–200 m-equiv/l. indicating sodium repletion.

Anaesthesia was induced by intravenous sodium thiopentone (0·3–0·5 g) and was continued, after tracheotomy, by tracheal tube with closed-circuit cyclopropane and oxygen. In all instances the animal remained in good condition throughout the course of the experiment.

Cannulation of the parotid duct, the preparation of the parasympathetic nerve (Moussu's nerve) for stimulation, and vascular isolation of the parotid gland have all previously been described (Coats, Denton, Goding & Wright, 1956). In all instances the sympathetic trunk was divided in the neck during the course of the preparation.

Stimulation of Moussu's nerve was carried out by means of a platinum cathode inserted into a chamber of Ringer–Tyrode solution surrounding the nerve. An indifferent brass electrode inserted into the rectum was used to complete the circuit. A square-wave electronic stimulator was used and maximum stimulation was attained with a voltage of 8–12 V, a pulse duration of 6 msec and a frequency of 22 pulses per second. For any one episode stimulation was continuous and saliva and venous blood were collected for consecutive periods of 10, 10, 20, 40 and 40 sec and in some instances a further 2 min. In three experiments further blood and saliva samples were taken at periods of up to 42 min after the beginning of stimulation.

In order to obtain volumes of saliva and venous blood adequate for chemical analysis during short period stimulation, the gland was rested for at least 5 min after each stimulation and was then restimulated at the same intensity and frequency, with pooling of the saliva and venous blood obtained from the relevant periods of each episode. In order to avoid the possibility of sampling error, saliva and venous blood were collected throughout the entire extent of any stimulation period and the chemical analyses were performed on a portion of the total sample. As previously described, the cannula dead space was estimated and allowance for this was made in timing the collection of all samples.

The chemical methods used were those described by Denton (1956).

RESULTS

Rate of flow

The rate of salivary production was measured by collecting into a graduated tube the saliva produced in each period. Venous outflow from the gland was measured directly by occluding the jugular vein (which carried only the venous drainage from the isolated gland) and simultaneously releasing a cannula previously inserted into a side tributary between the parotid gland and the point of jugular occlusion. Arterial inflow was calculated as the sum of salivary and venous outflows. This calculation is subject to two sources of error: it presupposes that during the course of stimulation the water content of the gland remained constant and that there was no lymphatic drainage from the gland. Over the period of time involved and with the rates of venous outflow observed, the first of these sources of error is negligible, but the second would cause all estimates of arterial inflow to be low.

Salivary and arterial flows

Following maximal motor nerve stimulation salivary flow rose to 5·80 ± 1·09 ml./min in the first collection period and fell progressively to achieve a steady flow of 2·76 ± 0·98 ml./min after continuous stimulation for 2 min. Arterial inflow, however, in all instances except one (A.E. 70) was maximal after stimulation of the gland for 2 min when the mean flow was 40·7 ± 6·9 ml./min. During the first 2 min of gland stimulation, arterial inflow was initially 36·1 ± 6·2 ml./min, and then fell after 20–40 sec to 31·7 ± 5·7 ml./min before rising progressively to achieve a steady flow of 40·7 ± 6·9 ml./min some time after stimulation for 2 min. In most instances (five out of seven) the arterial inflow was ten times the salivary outflow about 80 sec after the commencement of stimulation, and in the steady state the arterial inflow was about fourteen times the salivary flow. These relationships are shown in Fig. 1.

Because of this fairly constant pattern of arterial and salivary flows following stimulation of the gland, it was decided to test the hypothesis that salivary flow and composition, involving the dispersal of intraglandular substance, was in some way related to and dependent on the rate of arterial inflow. In order to do this it was necessary to modify the pattern of arterial flow during stimulation of the gland. The infusion of bradykinin is known to increase blood flow through tissues (Hilton & Lewis, 1955*a*, *b*). Augmented blood flow was induced in two animals by infusing bradykinin, at rates of 300, 480 and 675 μg/min, into the arterial blood perfusing the gland. Bradykinin infusion alone resulted in an increase of blood flow through the gland from 8–10 ml./min (basal flow) to 19, 38, and 14 ml./min, respectively. While the bradykinin was still running the glands were maximally stimulated and there occurred a further increase in blood flow to 50, 61 and 49 ml./min, respectively, during the first 10 sec of stimulation. Fig. 2 shows that while bradykinin completely altered the pattern of blood flow through the maximally stimulated gland and caused the initial blood flow to be the highest recorded, it was quite without effect on the pattern of salivary flow. The concurrent infusion of bradykinin did not significantly alter the composition of the saliva produced during maximal stimulation of the gland. It would seem that although both salivary flow and blood flow followed a fairly constant pattern during stimulation of the gland, the salivary flow is nevertheless a prime function of the stimulation and is not a function of the stimulation-induced hyperaemia. Bradykinin alone in these experiments did not cause a maximal blood flow through the gland.

Composition of saliva and blood

Throughout the course of maximal stimulation of the gland the salivary concentrations of sodium, potassium, chloride, bicarbonate and phosphate altered in the manner previously reported for Phase I and Phase II salivary flows (Coats & Wright, 1957). It was found that during the course of stimulation venous concentrations of these ions were significantly different from the arterial concentrations. This difference occurred during the first 2 min of stimulation (corresponding to the Phase I salivary flow) and thereafter, while stimulation continued, the venous concentrations more closely approached or became equal to the concentrations in the arterial inflow.

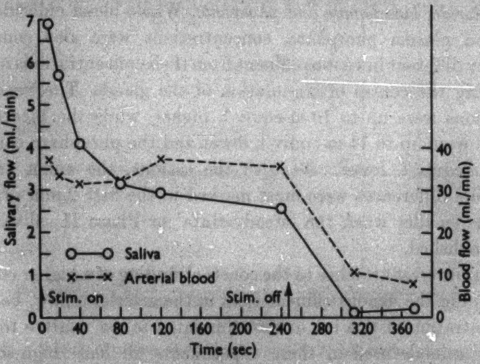

Fig. 1. The relationship between the rates of arterial inflow and salivary outflow. Eighty seconds after the commencement of maximal stimulation of the gland, arterial inflow was ten times salivary flow and in the steady state (Phase II) it was fourteen times salivary flow.

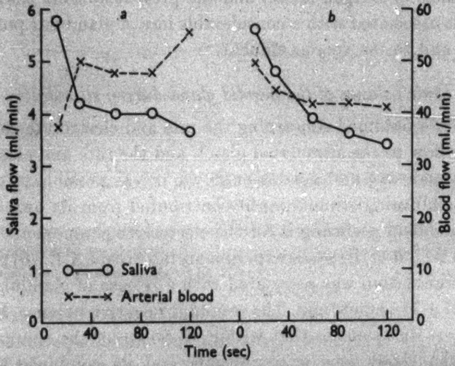

Fig. 2. Salivary and arterial flows during the first 120 sec of maximal gland stimulation (*a*) without bradykinin and (*b*) in the course of bradykinin infusion at 300 μg/min. The infusion of bradykinin has completely altered the pattern of arterial inflow without influencing the rate of salivary secretion.

Blood sodium and potassium. During the first 2–3 min of stimulation (Phase I) the whole-blood sodium concentrations in the venous effluent were constantly lower (1–15 m-equiv/l.) than in the arterial inflow, while whole-blood potassium concentrations in the venous effluent were constantly higher (up to 2·7 m-equiv/l.). During the ensuing 2–4 min (Phase II) this relation held for sodium but the concentration of potassium in the venous effluent closely approached, or was equal to, the arterial concentration.

Blood chloride, bicarbonate and phosphate. Whole-blood chloride and bicarbonate, and plasma phosphate, concentrations were also constantly and significantly different in venous effluent from their concentrations in the arterial inflow during the course of stimulation of the glands. The venous chloride concentrations were up to 10 m-equiv/l. higher, while the bicarbonate concentrations were up to 14 m-equiv/l. lower and the phosphate concentrations were 1–4 m-equiv/l. lower. As with the cations, the anion arteriovenous concentration differences were most marked in the first 2 min of stimulation and became smaller when the 'steady-state' or Phase II salivary flow had become established.

Particular interest attaches to the constant finding of a higher concentration of potassium in the venous effluent than in the arterial inflow, because of the high concentration of this ion in the stimulated saliva relative to that in the blood. The animals used in these experiments all had 'high-sodium, low-potassium' erythrocytes, and the highest whole-blood potassium concentration recorded was 11·0 m-equiv/l., whereas the highest salivary potassium concentration was 18·2 m-equiv/l. This is clear evidence confirming the previously reported findings of Burgen (1956) and the present authors that stimulation of the gland is associated with a considerable loss of glandular potassium both to the saliva and to the venous effluent.

Ionic balance of the parotid gland during stimulation

From the data obtained concerning the rate and electrolyte composition of the arterial inflow to the stimulated gland, and the rate and electrolyte composition of its salivary and venous outflows, it was possible to calculate the degree to which during stimulation it contributed from its own substance or drew from the blood perfusing it for the electrolyte components of its saliva.

Sodium. In six out of the seven experiments (all except A.E. 70) the first 10 sec of maximal stimulation was associated with a release of glandular sodium at the rate of 147 ± 78 μequiv/min. The resultant negative balance, however, was usually of short duration, so that within 40–80 sec of the commencement of stimulation the gland was in equilibrium and its combined salivary and venous output no longer exceeded its arterial delivery. In two animals, however (A.E. 63, 64), there was a further period of negative sodium balance between 120 and 240 sec before final equilibrium was achieved. The form of the initial sodium loss and the subsequent achievement of equilibrium is illustrated in Fig. 3.

Potassium. Of the electrolytes studied in this context potassium was the most consistent in its behaviour. During the first 10 sec of stimulation there occurred a glandular loss at the rate of 85 ± 30 μequiv/min, and in successive collections during the first 120–240 sec of stimulation this glandular loss reduced progressively until equilibrium was achieved. The initial 'explosive' release of potassium from glandular substance was less than in the case of sodium, but the duration of glandular loss before equilibrium was established was considerably longer. The form of the initial negative potassium balance during stimulation and its gradual decline towards equilibrium is illustrated in Fig. 4.

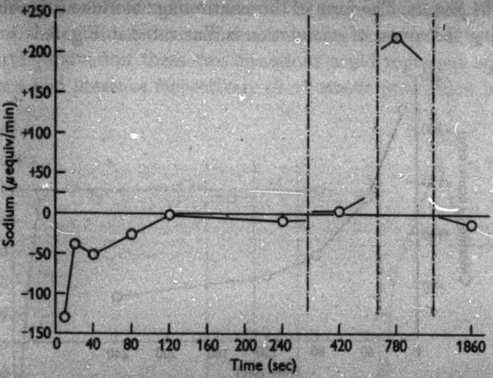

Fig. 3. The glandular balance of sodium at various times up to 31 min during maximal stimulation. During the first 10 sec of stimulation the gland is losing sodium at a rate of 130 μequiv/min, but equilibrium has been achieved by 120 sec and there is evidence of glandular accumulation of sodium at 13 min.

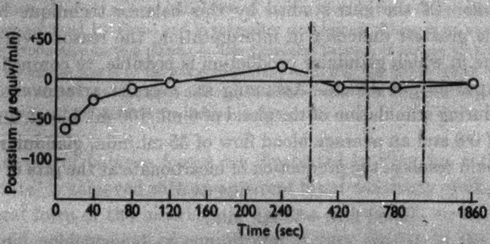

Fig. 4. The glandular balance of potassium at various times up to 31 min during maximal stimulation. During the first 10 sec of stimulation the gland is losing potassium at a rate of 63 μequiv/min, but equilibrium has been achieved by 120 sec and is thereafter maintained.

Chloride. In every case the first 10 sec stimulation of the gland was associated with a glandular accumulation of chloride. The subsequent behaviour of this ion as stimulation continued was variable but, in all cases except A.E. 68 (in which the total deficit was 13 μequiv), a continuing accumulation within the gland occurred, so that after stimulation for 2 min the mean chloride gain was 198 ± 54 μequiv. The form of this continuing chloride accumulation in the gland during the course of stimulation is illustrated in Fig. 5.

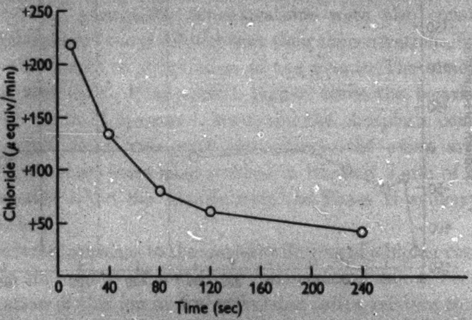

Fig. 5. The glandular balance of chloride at various times up to 240 sec during maxima lstimulation. There is a continuing accumulation of chloride at rates falling progressively from 224 μequiv/min at 10 sec to 42 μequiv/min at 240 sec.

Bicarbonate. Of the ions studied by this balance technique bicarbonate presents the greatest difficulty in interpretation, the reason being that it is the only one in which glandular production is possible, to compensate for an external input-output deficit. Assuming an average arteriovenous oxygen difference during stimulation of the gland of 6 ml./100 ml. blood, a respiratory quotient of 0·8 and an average blood flow of 35 ml./min, glandular metabolic activity would result in the production of bicarbonate at the rate of 75 μequiv/min:

$$\text{Blood flow} = 35 \text{ ml./min},$$

$$\text{oxygen used} = \frac{6 \times 35}{100} \text{ ml./min},$$

$$\text{CO}_2 \text{ produced} = \frac{0{\cdot}8 \times 6 \times 35}{100} \text{ ml./min},$$

$$22{\cdot}4 \text{ ml. CO}_2 = 1000\ \mu\text{equiv bicarbonate},$$

hence,

$$\text{CO}_2 \text{ produced} = \frac{0{\cdot}8 \times 6 \times 35 \times 1000}{22{\cdot}4 \times 100}\ \mu\text{equiv/min}$$

$$= 75\ \mu\text{equiv/min}.$$

For this reason any external negative bicarbonate balance of less than 75 μequiv/min may be apparent rather than real.

In all experiments there was an initial (apparent) bicarbonate loss of 270 ± 103 μequiv/min. This initial large loss, however, was followed within 40–80 sec by a steady state in which there was either no apparent further loss (A.E. 63, 68, 70) or a loss in the region of 100 μequiv/min (A.E. 62, 64, 65, 69). In view of the considerations above concerning the possibility of metabolic bicarbonate production these two situations could represent equilibrium in the absence and presence respectively of metabolic production by the gland.

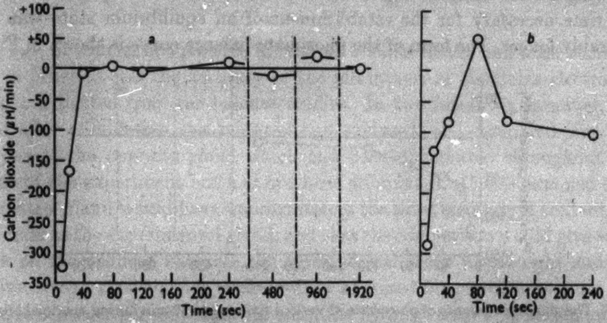

Fig. 6*a*. The glandular balance of carbon dioxide at various times up to 32 min during maxima stimulation. There is a large initial loss of carbon dioxide from the gland but equilibrium has been achieved by 40 sec and is thereafter maintained. There is no evidence of carbon dioxide production in this gland. *b*. The glandular balance of carbon dioxide in a gland in which the initial loss is of the same magnitude as in Fig. 6*a*, but in which a steady state is achieved with an apparent input deficit of 92 μequiv/min. This may be interpreted as evidence of metabolic carbon dioxide production in this gland.

The two types of balance found are illustrated in Figs. 6*a*, *b* (in which the 'steady state' is achieved with an apparent input deficiency of 92 μequiv/min). The general form of the bicarbonate balance curves with an initial large loss and a rapid achievement of equilibrium is very similar to the form of the sodium balance curves.

Phosphate. For technical reasons phosphate analyses were performed on arterial and venous plasma, whereas for the other ions studied the analyses were performed on whole blood. For that reason the phosphate balance of the gland throughout the course of stimulation must be presented with less assurance than the balances of the other ions. Differences in the haematocrit values of arterial and venous blood and the possibility of movement of phosphate between red cells and plasma, either during the course of the passage of the blood through the gland or during the interval between blood sampling and plasma separation, may all impose limits upon the validity of the phosphate balances. It is probable that the errors introduced by this technique were small relative to the glandular depletion of phosphate which occurred but their possible presence should be appreciated.

In all cases there was an initial loss during the first 10 sec of stimulation of 72 ± 37 μequiv/min. This loss became progressively smaller during subsequent collection periods until equilibrium was achieved between 120 and 240 sec after the beginning of stimulation. The initial 'explosive' release of phosphate from gland substance was smaller than in the case of bicarbonate (even allowing for the complicating factor of possible bicarbonate production), but the time necessary for the establishment of an equilibrium state was considerably longer. The form of the phosphate-balance curve is shown in Fig. 7. It is similar to the potassium curve (Fig. 4) with an initial loss smaller than in the case of sodium or bicarbonate but a longer period before the establishment of equilibrium.

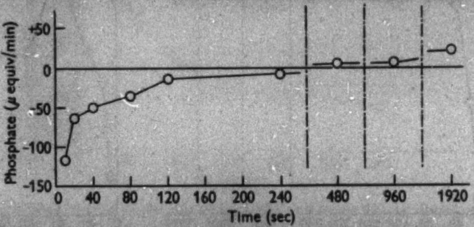

Fig. 7. The glandular balance of phosphate at various times up to 32 min during maximal stimulation. During the first 10 sec of stimulation the gland is losing phosphate at a rate of 118 μequiv/min, but equilibrium has been achieved by 120–240 sec and is thereafter maintained.

Cumulative balances. Cumulative balance studies on the glands in these experiments revealed that during the course of the first 2 min of maximal stimulation there was always a glandular loss of potassium and bicarbonate (although a bicarbonate correction for metabolic production should probably be applied in some cases). In all cases except one there was also a sodium loss, a phosphate loss and a chloride accumulation in the gland. These cumulative 2 min balances are presented in Table 1.

Where the total cation loss is greater than the total measured anion loss endogenous bicarbonate production can probably be invoked to explain the discrepancy. In three experiments, however (A.E. 62, 63, 69), cation loss is associated with an apparent accumulation of anion. In these cases the total cation loss is of greater magnitude and the disturbance in ionic state may well have been sufficient to alter significantly the pH and anion equivalance of intracellular protein and organic phosphate.

TABLE 1. Cumulative ionic balances after 2 min maximal stimulation of parotid gland (μequiv)

						Net gland state	
Expt.	Na^+	K^+	Cl^-	CO_2	HPO_4^-	Cation	Anion
A.E. 62	−140·4	−108·9	+116·8	− 71·7	− 15·1	−249·3	+ 30·0
A.E. 63	−129·6	− 71·6	+177·2	− 22·6	−117·5	−201·2	+ 37·1
A.E. 64	−204·3	−109·4	—	−158·6	—	−313·7	—
A.E. 65	− 64·9	− 31·8	− 92·0	−239·5	+ 6·1	− 96·7	−141·4
A.E. 68	+ 9·1	− 64·2	− 12·7	− 90·5	− 80·6	− 55·1	−183·8
A.E. 69	− 53·7	−116·9	+553·9	−124·2	− 51·3	−170·6	+378·4
A.E. 70	+280·8	− 99·4	+261·0	− 55·3	− 28·0	+181·4	+177·7

Gland analysis

It was, of course, not possible to analyse a gland before and after stimulation in order to test the accuracy of the alterations in glandular electrolyte content indicated from the balance studies. In two instances, however, the gland after stimulation was removed and analysed, and compared with the analysis of the opposite gland which had been denervated throughout the course of the experiment but had not been stimulated. It was assumed that the control gland would have approximately the same electrolyte content per unit mass as the experimental gland, and that the comparison would give some check on the deduced balances. In three normal sheep whose right and left unstimulated parotid glands were similarly analysed the paired potassium and chloride contents per unit mass did not differ by more than 5%, and in two of them the sodium content on each side also was within 5%. In the third there was a difference of 10% in the sodium content per unit mass of gland on the two sides. The analyses gave an indication that the stimulated glands had lost potassium and had gained both sodium and chloride and that the magnitude of the deduced potassium loss (200 μequiv) was feasible (analysis deficit for the same gland 164 μequiv when compared with the same mass of the opposite unstimulated gland).

Ionic fluxes across the secretory cell

In view of the rapidity of ionic transference to saliva during maximal stimulation of the gland an attempt was made to determine its order of magnitude. Histological study of the gland showed that the mean outside acinar diameter was of the order of 20 μ and the mean inside acinar diameter was of the order of 5 μ. Analyses of normal glands allow an assumption of an extracellular water content of the gland of 50% of its mass and it was assumed that the specific gravity of the acini did not differ significantly from unity. The maximum transferences of sodium, potassium and chloride and average transferences obtained during Phase II salivary flows are indicated in Table 2.

In computing the transferences it was assumed that each acinus was a sphere, that there was no secretion from the duct system and that the acinus did not contain any secretory canaliculi. These assumptions should be valid for the external (blood to acinar cell) transfer, but may not be valid for the internal (acinar cell to saliva) transfer. Should the assumptions be invalid the magnitudes of internal transference would be rather less than those indicated.

In the case of potassium the initial explosive release from the gland following stimulation involves dispersal to venous effluent as well as to saliva. The mean magnitude of this potassium flux to venous blood for the first three collection periods following the commencement of stimulation (10, 10 and 20 sec) is indicated in Table 3.

TABLE 2. Electrolyte transferences to saliva (*a*) during peak flow following stimulation and (*b*) during established (Phase II) secretion

Ion	Concn. (m-equiv/l.)	Salivary flow (ml./min)	Transference across external acinar membrane (pmoles/cm²/sec)	Transference across internal acinar membrane (pmoles/cm²/sec)
Na^+ (peak flow)	190	6·9	$2{\cdot}5 \times 10^3$	$4{\cdot}0 \times 10^4$
Na^+ (Phase II flow)	182	1·35	$5{\cdot}0 \times 10^2$	$8{\cdot}0 \times 10^3$
K^+ (peak flow)	14·3	6·9	$1{\cdot}9 \times 10^2$	$3{\cdot}0 \times 10^3$
K^+ (Phase II flow)	9·8	1·35	$2{\cdot}5 \times 10$	$4{\cdot}0 \times 10^2$
Cl^- (peak flow)	25	7·2	$3{\cdot}4 \times 10^2$	$5{\cdot}4 \times 10^3$
Cl^- (Phase II flow)	22	1·35	$6{\cdot}0 \times 10$	$9{\cdot}6 \times 10^2$

TABLE 3. Net potassium flux from acinar cells to venous blood at the beginning of stimulation

Period (sec)	K^+ flux (pmoles/cm²/sec)
0–10	17
10–20	42
20–40	17

DISCUSSION

The denervated parotid gland of the sheep maintains a continuous basal secretion of saliva in the absence of external stimuli. In this phase the electrolyte content of the gland presumably remains constant and the sole source of salivary components must be the perfusing blood. When the gland is stimulated, however, there occurs not only an increase in the rate of production of saliva, but also a complex readjustment of its ionic constituents associated with an alteration in the ionic content of the gland. During this phase of altering secretory activity there is no longer a balance between the arterial inflow of ions and their combined salivary and venous outflows. The present work has shown that the imbalance is characterized by a dispersal of glandular sodium, potassium, phosphate and carbon dioxide and an accumulation of chloride. In all instances the magnitude of the imbalance is greatest during the first 10 sec of stimulation and thereafter gradually declines towards a re-set equilibrium between inflow and outflow. This re-setting of the equilibrium, however, does not imply a restitution of the gland to its basal (denervated and unstimulated) constitution.

The dispersal of glandular substance and the re-setting of equilibrium during stimulation do not occur over the same period of time for all the ions studied. In general there are two main orders of magnitude. At the beginning of stimulation there occurs a sudden and 'explosive' efflux of glandular sodium and bicarbonate with glandular depletion proceeding at the rates of 147 ± 78 and 270 ± 103 μequiv/min, respectively (the apparent bicarbonate depletion may, however, be spuriously high owing to concurrent carbon dioxide production within the gland). These large initial negative balances are of relatively short duration and within a period of 40–80 sec of continuous stimulation equilibrium has been achieved (allowing for the possibility of carbon dioxide production by the gland) with near equality of net arterial input and salivary loss. The second order of intrinsic glandular depletion following stimulation is seen with the ions potassium and phosphate. Here the initial losses are considerably smaller (85 ± 30 and 72 ± 37 μequiv/min, respectively) but the time interval required before equilibrium is achieved is of the order 120–240 sec. Equilibrium, of course, does not involve restitution of the gland to its pre-stimulation ionic composition. In three experiments (A.E. 64, 65, 68) stimulation was maintained for a total period of 32–42 min and the cumulative balances indicated in each instance a total glandular deficit of potassium and excess of sodium compared with the resting state. Whether resting glandular composition is ever regained during continuous stimulation is not known.

The chloride content of the stimulated gland is of interest in that in all experiments except one (A.E. 68) there occurred a glandular accumulation of chloride during stimulation. During the first 10 sec this accumulation was at the rate of 141 ± 62 μequiv/min and at the end of 2 min of stimulation the total chloride accumulation was 198 ± 54 μequiv. One explanation might be that stimulation of the gland resulted in oedema with an increased extracellular chloride content. This, however, would require a concomitant sodium accumulation of some 280 μequiv to maintain extracellular proportions of the two ions, whereas the corresponding mean sodium balance was a deficit of 16 ± 96 μequiv. This must mean that the chloride accumulation was intracellular, a conclusion supported by histological evidence of lack of significant glandular oedema following stimulation.

Consideration of the 'explosive' glandular release of cellular substance at the beginning of stimulation suggests the possibility that the requirements of ions for secretion in saliva at this stage is in excess of arterial delivery. This might particularly be so in the case of potassium and phosphate where the arterial plasma concentrations are considerably below the salivary concentrations and in the first few seconds of stimulation when salivary volume is maximal and arterial inflow is still rising (Fig. 2). Should arterial inflow at the beginning of stimulation impose a limit on the ions from this source available for secretion then glandular contribution of the remainder would be explicable.

This hypothesis was tested by increasing blood flow through the gland by the infusion of bradykinin before stimulation. This resulted in a maximal blood flow associated with the first salivary collection without altering the 'explosive' release of glandular substance or the rate of salivary production. This evidence would indicate that the release of glandular substance into saliva at the beginning of stimulation was a prime function of the stimulation rather than a reflexion of paucity of external supply. The conclusion is further supported in the case of potassium by the constant finding at the beginning of stimulation of a higher concentration in venous effluent, than in arterial inflow indicating a glandular contribution both to saliva and blood.

TABLE 4. Fluxes across various biological membranes (pmoles/cm^2/sec)

	Ion	Influx	Efflux	Net flux	Reference
Sepia axon (post-stimulation)	Na^+	32	39	—	Hodgkin & Keynes (1955)
	K^+	21	28	—	
Sepia axon (resting)	Na^+	61	31	—	Keynes (1951)
	K^+	17	58	—	
Desheathed cat nerves					
(If whole surface is available for exchange)	Na^+	—	3·9	—	Dainty & Krnjević (1955)
(If only region near nodes of Ranvier is available for exchange)	Na^+	—	500–2500	—	
Frog skin (short-circuited)	Na^+	514	20	494	Ussing & Zerahn (1951)
Human kidney tubule	Na^+	—	—	3000	Rehberg (1926)
Cat					
Submaxillary gland	K^+	—	30–40	—	Lundberg (1958)
Sublingual gland	K^+	—	400–500	—	
Sheep parotid					
Maximal response	Na^+	—	—	2500 (plasma to cell) 40,000 (cell to duct)	This paper
Steady flow	Na^+	—	—	500 (plasma to cell) 8000 (cell to duct)	
Maximal response	K^+	—	—	190 (plasma to cell) 3000 (cell to duct)	
Steady flow	K^+	—	—	25 (plasma to cell) 400 (cell to duct)	

The magnitudes of the ionic fluxes across the secretory cell during stimulation are very large compared with those of the fluxes for stimulated Sepia axons (Keynes, 1951; Hodgkin & Keynes, 1955) and for desheathed cat nerves (Dainty & Krnjević, 1955). Ussing & Zerahn (1951) showed a sodium influx across short-circuited frog skin of 1·85 μmoles/cm^2/hr (514 pmoles/cm^2/sec) and Rehberg (1926) gives a figure of 3000 pmoles/cm^2/sec for the net sodium flux across human renal tubular cells. Table 4 presents a summary of representative fluxes of sodium and potassium taken from the literature compared with the net fluxes found for the sheep's parotid gland. Only the figures of Dainty & Krnjević (1955) for sodium fluxes for the desheathed cat nerve are in any way of comparable magnitude to those here presented and then only if the assumption is made that exchange is limited either to the myelin-free gap or to a 10 μ length in the region of the node of Ranvier. The authors give reasons for rejecting this hypothesis of restricted area of exchange. In a recent publication Lundberg (1958) quotes from unpublished work of Krnjević & Lundberg an initial potassium efflux in the submaxillary gland of the cat of 30–40 pmoles/cm^2/sec and in the sublingual gland of about 400–500 pmoles/cm^2/sec.

SUMMARY

1. Stimulation of the parotid gland of the sheep gives rise not only to a great increase in the rate of salivary production, and changes in salivary composition which may be divided into two phases, but also to an 'explosive' release of glandular ionic constituents which appear in the saliva. There is a large negative balance of sodium and bicarbonate which persists for the first 40–80 sec of stimulation before equilibrium is achieved. There is also a smaller negative balance of potassium and phosphate which, however, persists for the first 120–240 sec of stimulation. During the period of glandular depletion of these ions there occurs a glandular accumulation of chloride. The negative balance of potassium is reflected not only in the loss of glandular potassium to saliva but also in its dispersal into the venous effluent from the gland.

2. After the period of glandular imbalance at the beginning of stimulation a new equilibrium is achieved in which there is an equality of arterial input and combined salivary and venous outflows of the ions sodium, potassium, chloride, bicarbonate and phosphate. This new equilibrium is achieved during continuing stimulation without restitution of the gland to its basal (pre-stimulation) composition. After continuous stimulation for a period of 32–42 min, the gland has a higher content of sodium and chloride and a lower content of potassium than the unstimulated gland.

3. The release of glandular content during the initial phase of stimulation is not due to a lack of availability of ions for secretion in the arterial blood perfusing the gland. Increasing the blood flow by the infusion of bradykinin before and during stimulation increases the availability of ions for diversion into the saliva, but fails to modify the 'explosive' dispersal of glandular substance or the composition and rate of production of saliva.

4. Ionic fluxes across the acinar cells are very large when compared with fluxes across nerve, frog skin and renal tubules. The highest fluxes occur during maximal stimulation of the gland, and across the internal (acinus to saliva) acinar membrane the order of magnitude for sodium, potassium and chloride

is $4\cdot0\times10^4$, $3\cdot0\times10^3$ and $5\cdot4\times10^3$ pmoles/cm^2/sec, respectively. During established (Phase II) salivary flow while stimulation continues the corresponding fluxes for the same ions are of the order of $8\cdot0\times10^3$, $4\cdot0\times10^2$ and $9\cdot6\times10^2$ pmoles/cm^2/sec, respectively. In all cases the ionic fluxes across the external (blood to acinus) acinar membrane are smaller by a factor of 16.

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GASTRIC SECRETORY RESPONSES AFTER PORTAL VENOUS LIGATION

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It was shown by Lebedinskaya (1932) that the creation of an Eck fistula in dogs provided with Pavlov pouches was followed by a remarkable change in the secretory response to meals. The volume and acidity of the juice increased greatly and the duration of secretion was prolonged; the output of pepsin was slightly decreased. These changes attained their maximal extent about four weeks after the operation and were apparently permanent thereafter. She believed that they were due to an impairment of the detoxicating functions of the liver which resulted from the diversion of its portal blood supply into the systemic circulation, and saw in her results a possible explanation for clinical reports that gastric hypersecretion occurred in cirrhosis of the liver.

Gerez & Weiss (1936) repeated Lebedinskaya's experiments and obtained virtually identical results. They, however, referred to reports that secretin extracts were less effective in stimulating pancreatic secretion when injected into the portal blood stream than when they were injected into a peripheral vein, suggesting that some retention or destruction by the liver normally occurred during passage of the hormone through this organ. Gerez & Weiss suggested that this was true also for stimulants of gastric secretion released into the portal blood during digestion of a meal, so that after creation of an Eck fistula they appeared in greater quantity in the systemic blood during digestion.

These observations are of great potential significance in relation to the mechanisms by which gastric secretion is stimulated during digestion in normal circumstances; but before any useful interpretation can be made of them from this point of view further experimental analysis is necessary, if only for the following reasons: (1) the gastric pouches used in the above experiments were vagally innervated, so that the extent of humoral stimulation before and after the creation of the Eck fistula cannot be estimated; and (2) it is generally agreed that the liver of the Eck fistula dog undergoes considerable atrophy, with associated impairment of functional reserve (Bollman & Mann, 1936); and the influence of this upon the results described is problematical.

It therefore seemed of value to study further the effects on gastric secretory responses of diverting portal venous blood into the systemic circulation. In order to exclude nervous influences, the gastric pouches used were completely denervated; and in order to minimize the impairment of liver function likely to result from porto-caval diversion of blood, the method recently described by Grindlay & Mann (1952) was adopted. This consists of partially ligating the portal vein above its highest tributary with cellophane tape. In a few weeks after the operation, fibrosis of the vein wall beneath the ligature practically completes its closure; and portal venous blood is thus diverted through collateral channels into the systemic circulation. Although the end result corresponds closely to that of the Eck fistula, no structural changes occur in the liver comparable with those usually observed in that preparation, and the animals never show the classical condition of 'meat intoxication' (Hahn, Massen, Nencki & Pavlov, 1893) which is probably a consequence of advanced impairment of liver function.

A preliminary account of the results presented in this paper has been given to the Physiological Society (Gregory, 1957).

METHODS

All the dogs used in these experiments were provided with denervated gastric pouches fitted with water-tight silver cannulae of the pattern first described by Gregory (1950). The pouches were made in the usual way from the greater curvature of the stomach and so were supplied by blood vessels running from the hilum of the spleen. This organ was removed by ligation of the vessels as close as possible to the hilum, so that the blood supply from the splenic vessels to the pouch was not interrupted; and the splenic artery and vein were then traced towards their origins, where they were cleaned of all connective tissue and nerves for a distance of about 2 cm. In this region denervation without damage to the vessels involved is easy because of their large size and because of the collection of the nerves into a few large bundles which are readily removed.

The spleen was removed during preparation of the pouch for two reasons. First, this reduces (probably by as much as 30%) the blood to be carried by the collateral vessels after portal venous ligation, which is obviously of advantage; and secondly, it was anticipated that if not removed the denervated spleen might influence the secretory responses of the pouch, for both are supplied 'in parallel' by the splenic artery. It is known that the denervated spleen is supersensitive to circulating humoral agents; and relaxation of it, or dilatation of its vessels, might shunt blood away from the pouch.

Six dogs were provided at the same operation with a wide-bore water-tight silver gastric cannula installed in the body of the stomach and brought out through the mid line. In two dogs prepared early in the series, the second operation of portal venous ligation (see below) was performed and observations subsequently made without provision of a gastric cannula; and in two dogs prepared towards the end of the experiments, when the general pattern of the results was established, the entire preparation (denervated pouch, gastric cannula, splenectomy, and ligation of portal vein) was carried out at the same operation. In the six dogs referred to above, ligation of the portal vein was carried out at a second operation, performed some weeks after the first. The directions of Grindlay & Mann (1952) were followed closely and the same material used. I am indebted to Dr J. H. Grindlay for a generous supply of the laminated tape used in his experiments. The degree of constriction imposed was rather greater than that described by the above authors, corresponding to about four-fifths of the original diameter of the vessel as seen after cleaning. This greater initial degree of constriction resulted in a more rapid development of the secretory changes to be described below, and was probably made possible without mishap by the previous splenectomy. After applying the ligature, the abdomen was not closed for 5–10 min. If during this time obvious cyanosis of the intestines became apparent, the ligature was removed and another applied slightly more loosely. The complete preparation is represented diagrammatically in Fig. 1.

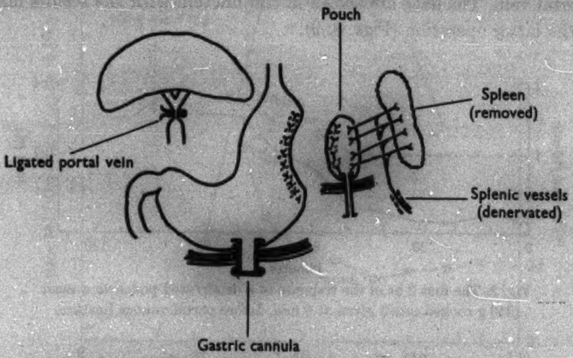

Fig. 1. The features of the complete preparation: ligated portal vein, gastric cannula, gastric pouch, splenectomy, denervation of splenic vessels.

In three dogs oesophagostomy was performed at a third operation for the purpose of sham-feeding. The technique of this operation and the regime of after-care was similar to that described by Gregory (1950). Maximal secretory responses of the pouches to histamine were determined at intervals before and after portal venous ligation by a procedure similar to that described by Code, Blackburn, Livermore & Ratke (1949). Mepyramine (May & Barker) was given in a single dose of 50 mg intravenously to minimize side effects during the tests. Determinations of peptic activity in gastric juice samples were made by Hunt's method (1948). Total acid in gastric juice samples was determined by titration to phenolphthalein using 0·1 N- or 0·02 N-NaOH.

RESULTS

Normal secretory responses of the denervated pouch

Meals. A typical response of the denervated gastric pouch to a meal (250 g cooked meat) is shown in Fig. 2. From the point of view of the present work, the significant feature of the response is that little or no stimulation of secretion is usually detectable for at least 15–30 min after feeding. Subsequently, the flow of juice gradually increases to a maximum 3–5 hr after the meal and slowly declines thereafter. When a larger meal is fed, the ultimate response is greater and lasts longer, but the general pattern is unchanged; there is little or no immediate response and the maximum rate of secretion is attained rather later than after a smaller meal. The maximal volume output and acidity of the juice secreted by such pouches in response to a meal is always very low and does not compare with that which the pouch is capable of producing in response to histamine.

The maximal secretory response to histamine. Following recovery from the first operation, at least three determinations of the maximal secretory response to histamine were made at approximately weekly intervals before ligation of the portal vein. The data are shown in conjunction with the results obtained after the latter operation (Figs. 8, 9).

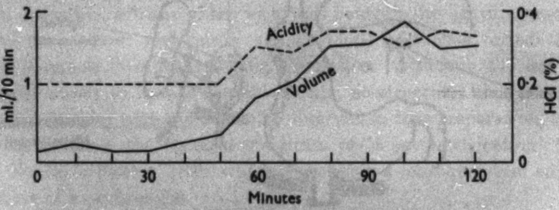

Fig. 2. The first 2 hr of the response of a denervated pouch to a meal (250 g cooked meat) given at 0 min, before portal venous ligation.

The effect of portal venous ligation

When satisfactory data for responses to meals and the maximal secretory response to histamine had been obtained on each dog, the portal vein was ligated (see 'Methods'). After recovery from this operation, the following observations were made.

Feeding. All the animals ate food readily when it was first offered 24 hr after operation; and it at once became apparent that the secretory response of the pouch was profoundly changed. The denervated pouch commenced to secrete juice of high acidity within 10 min of feeding and a considerable response was in progress by the end of the first 30 min after feeding. In all the dogs this response to feeding increased progressively in magnitude and rapidity of onset over a period of 3–4 weeks following the operation, until volume and acid outputs far greater than those observed before operation were attained even when the amount of meat fed was small, e.g. 100 g (Fig. 3); the pouches responded to meals as though they were vagally innervated. The total acidity of the juice secreted after meals rapidly approached that accepted as maximal for the dog (approximately 0·6 % (w/v) HCl; Gray, Bucher & Harman, 1941) and remained high even when the volume rate of secretion declined towards the end. It is doubtful whether any stimulation of pepsin output occurred; the concentration fell to low levels as secretion began and remained there (Fig. 3). However, although the size of the response to a meal was thus greatly increased, the duration of it was decreased. This is evident from Fig. 4, in which a large meal (500 g meat) was fed. The response was largely over in 1½ hr after feeding, the volume rate having fallen by that time to about one-fifth of its maximal value. This observation suggests that either gastric emptying is greatly accelerated after portal venous ligation, or that

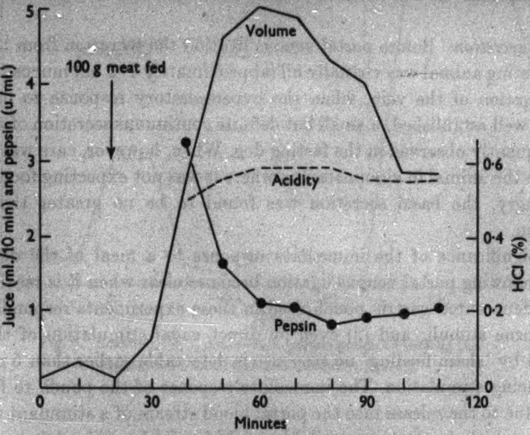

Fig. 3. Response of a denervated pouch to feeding 100 g cooked meat; 4 days after portal venous ligation.

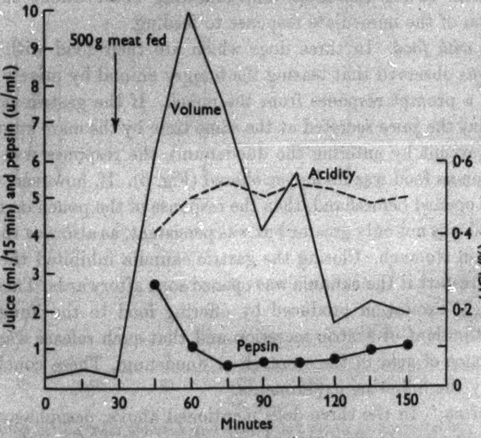

Fig. 4. Response of a denervated pouch to feeding 500 g cooked meat; 16 days after portal venous ligation.

the source of stimulation of secretion is gastric rather than intestinal in origin. The experiments illustrated in Figs. 3–5 also show a feature which was commonly observed in the other animals, namely, the remarkable speed with which the maximal secretory rate in response to a meal was attained; on these occasions it was reached within 20–30 min after commencement of feeding.

Basal secretion. Before portal venous ligation the secretion from the pouch in the fasting animal was virtually nil (approximately 1–2 ml. mucus per hour). After ligation of the vein, when the hypersecretory response to meals had become well established, a small but definite continuous secretion of acid juice was frequently observed in the fasting dog. When, however, care was taken to examine the animal in circumstances where it was not expecting food and was not hungry, the basal secretion was found to be no greater than before operation.

The significance of the immediate response to a meal of the denervated pouch following portal venous ligation becomes clear when it is recalled that: (1) the denervated gastric pouch used in these experiments responds only to blood-borne stimuli; and (2) even on direct vagal stimulation of the intact stomach by 'sham-feeding' no response is detectable earlier than 5 min after commencing stimulation. The immediate response of the pouch to feeding is clearly due to the release into the portal blood stream of a stimulant of gastric secretion which, in the time available, could hardly be derived from digestion of the swallowed food, but must be liberated as a result of the vagal excitation associated with eating the meal. The following observations support this interpretation of the immediate response to feeding.

'Teasing' with food. In three dogs which ate their food with particular avidity, it was observed that teasing the hungry animal by merely offering it food caused a prompt response from the pouch. If the gastric cannula was closed, so that the juice secreted at the same time by the main stomach could not escape (except by entering the duodenum), the response was small and ceased as soon as food was no longer offered (Fig. 5). If, however, the gastric cannula was opened beforehand, then the response of the pouch on teasing the dog with food was not only greater but was persistent, as also was the secretion from the main stomach. Closing the gastric cannula inhibited the response; it tended to restart if the cannula was opened soon afterwards. This suggested that the vagal excitation produced by offering food to the hungry animal released a stimulant of gastric secretion and that such release was inhibited by the presence of acid in the stomach or duodenum. These conclusions are supported by the following experiments.

'Sham-feeding.' In the three dogs mentioned above, oesophagostomy was performed and sham-feeding carried out after recovery from the operation. A prompt and considerable response from both the main stomach and the

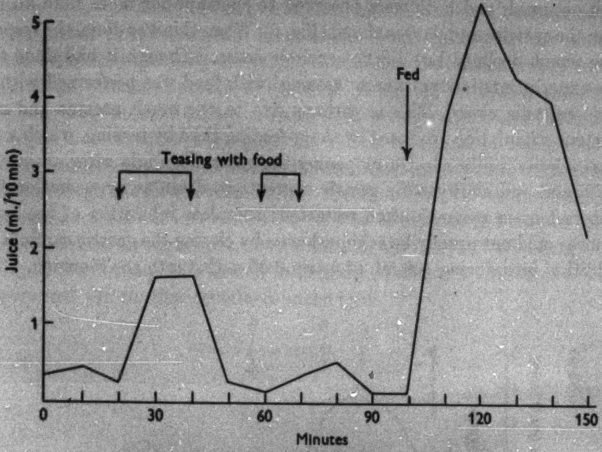

Fig. 5. Responses of a denervated gastric pouch to teasing the animal with food, and to subsequent feeding.

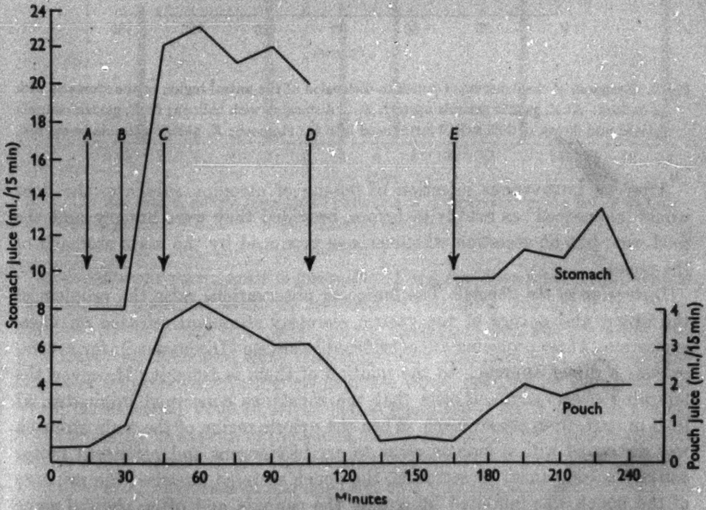

Fig. 6. Response of the main stomach and denervated pouch to sham-feeding, with open and closed gastric cannula; 9 days after portal venous ligation. *A*, gastric cannula opened; *B–C*, sham-feeding with meat; *D*, gastric cannula closed; *E*, gastric cannula opened.

pouch occurred, and both were observed to continue for more than an hour before the gastric cannula was closed (Fig. 6). When this was done, the response of the pouch declined but did not entirely cease, although it had done so in those experiments (above) where 'teasing' with food was performed with the gastric cannula closed. This is perhaps due to the much greater and more persistent stimulation produced by sham-feeding than by teasing, which would be less readily inhibited. On reopening the gastric cannula after an interval (30–60 min) and allowing the gastric contents to drain away, secretion from pouch and main stomach often restarted; complete inhibition of the pouch response could not usually be achieved even by closing the gastric cannula and in addition introducing 100 ml. of warm 0·05 N-HCl into the stomach.

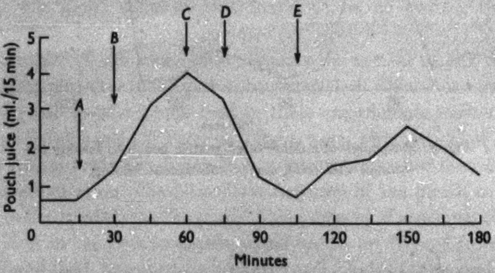

Fig. 7. Response of the denervated pouch to distension of the antral region of the stomach with a balloon. At *A*, gastric cannula opened; *B–C*, distension with balloon; *D–E*, gastric cannula closed and 50 ml. of 0·05 N-HCl introduced into the stomach; *E*, gastric cannula re-opened.

After an intravenous injection of 0·5 mg of atropine sulphate, the dogs would 'sham-feed' as readily as before, provided they were hungry and the food wet; but no secretion whatever was produced by the main stomach or the pouch.

Distension of the stomach. The foregoing observations raise the problem of the origin and nature of the gastric secretory stimulant released on vagal excitation. These problems are considered later (see 'Discussion'); for several reasons a direct approach to the solution of them is difficult. However, the following observation suggests that the substance concerned originates, at least in part, from the stomach. When the pyloric region of the main stomach was distended with a balloon so as to excite vigorous and persistent motor activity accompanied by secretion, the pouch secreted likewise; the response of the pouch was inhibited by closing the cannula and often showed some tendency to restart if the gastric cannula was opened again shortly afterwards (Fig. 7).

The balloon was made of condom rubber, mounted upon a Perspex bobbin, and connected to a bromoform manometer. When inflated to the maximal volume without appreciably stretching the rubber, the balloon was of toroidal form and its volume approximately 30 ml. A balloon of these dimensions in a dog of average size (10–20 kg) remains in the pyloric region and the stimulus it provides is confined to that region. The rubber tube must be tethered so that the balloon does not pass into the duodenum. After introduction of the deflated balloon through the gastric cannula into the stomach in a direction towards the pyloric antrum, air was introduced to give a basal pressure of 2 cm bromoform. In a few minutes strong regular contractions of the antrum were excited. They continued indefinitely and tended to increase in strength, although the basal pressure and the rhythm remained unchanged.

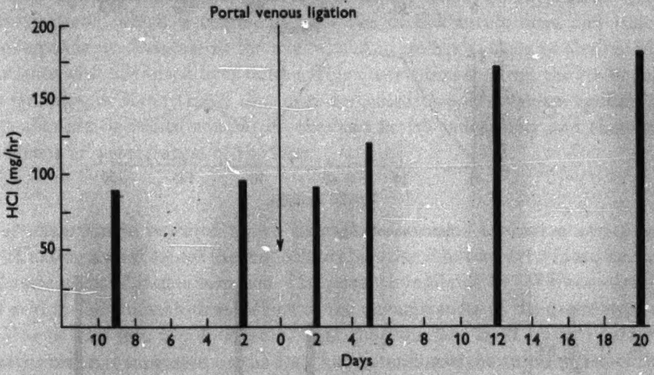

Fig. 8. The maximal secretory response to histamine of a denervated pouch before and after portal venous ligation.

Maximal secretory response to histamine. For a few days after portal venous ligation, the maximal secretory response to histamine was not found to be significantly different from that before operation; but shortly after this (5–7 days) an increased acid output became apparent, and the response increased progressively during the next 3–4 weeks until values were obtained which far exceeded those observed before operation (Figs. 8, 9). This change in the secretory behaviour of the pouch, like those already described in relation to meals etc., appeared to be permanent; they were undiminished in extent 2 months after operation in one dog, and at 6 weeks in another.

Post-mortem and general observations. Throughout the experiments reported here the dogs remained in excellent health and ate with undiminished appetite; a supplement of NaCl and KCl corresponding to the expected daily loss in the pouch secretion was added to the diet. At post-mortem, no ulceration of stomach or duodenum was found, but in one of the dogs which secreted most vigorously on feeding there was some inflammation of the duodenal mucosa for about 7 cm below the pyloric sphincter. No dilated veins were seen in any part of the abdominal viscera and the only evidence of a collateral circulation in the abdominal wall was some enlargement of the inferior epigastric veins. There were no vascular adhesions between the liver and other organs in any of the animals, and in every case the organ was of normal size and appearance.

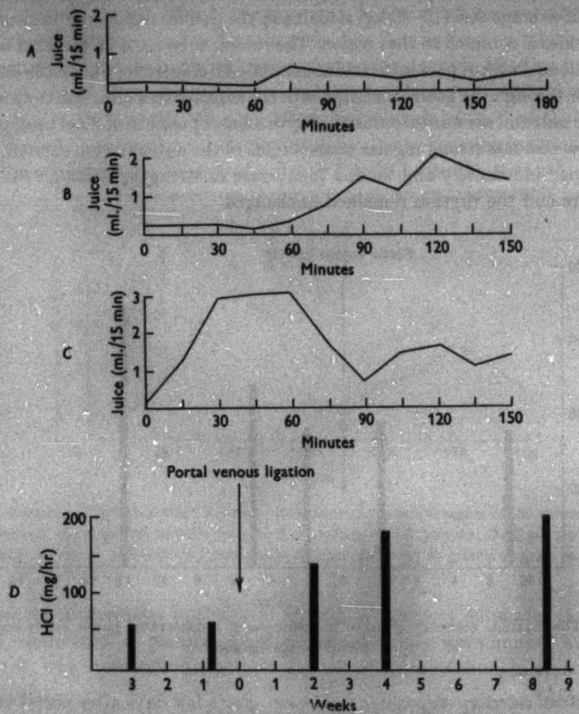

Fig. 9. The effect of portal venous ligation on the response to a meal and the maximal secretory response to histamine in a dog provided with a Heidenhain gastric pouch and previously antrectomized. *A*, meal response 1 day before portal venous ligation (meal at zero time); *B*, meal response 14 days after portal venous ligation (meal at zero time); *C*, meal response 28 days after portal venous ligation (meal at zero time); *D* maximal secretory response to histamine before and after portal venous ligation.

Histologically, the only changes observed in the liver were the presence in the biliary epithelium of cytoplasmic vacuoles and the occasional occurrence of small cytoplasmic droplets in the liver cells. I am indebted to Dr N. M. Hancox of this Department for making these observations for me.

In all the dogs except one the ligature was found intact on the portal vein embedded in fibrous tissue. Beneath the ligature the vein wall was appreciably thickened and the lumen practically occluded, although a minute free passage could always be found, and there was no thrombosis in the vein above or below the ligature. In the dog mentioned above as the exception, the expected changes in secretory activity of the pouch after the operation of portal venous ligation failed to materialize; an eventual laparotomy showed that the ligature had loosened and the portal vein was of normal size and patency.

The dogs described in this paper did not at any time show any sign of 'meat intoxication', which is commonly observed in Eck fistula dogs and which corresponds in character to the mental and neurological changes observed in patients with advanced liver failure. This observation confirms the statement of Grindlay & Mann (1952) that dogs subjected to portal venous ligation in the manner described remain in excellent health indefinitely and show no evidence of degeneration of the liver.

DISCUSSION

The experiments reported above show that the vagal excitation associated with eating a meal causes the immediate liberation into the portal blood stream of a stimulant of gastric secretion. This release is inhibited by the accumulation of acid in the stomach, from which in the circumstances of the experiment it is free to pass into the duodenum. That the stimulant is, at least in part, of gastric origin is suggested by the fact that distension of the antral region of the stomach with a balloon causes the denervated pouch to secrete.

In considering the further interpretation of these findings, it is necessary at the outset to exclude the possibility that a state of portal hypertension resulting from ligation of the portal vein plays an essential part in their production. No measurements of portal venous pressure were attempted in these animals; but it seems unlikely that portal hypertension of any significant degree existed, partly because the portal load was reduced by splenectomy and also because it appears to be difficult to produce persistent portal hypertension in dogs by ligation of the portal vein. In acute experiments on dogs Bergstrand & Ekman (1957) showed that following portal ligation the collateral vessels rapidly increase in size and the initial increase in portal pressure diminishes; and Morris & Miller (1951) did not succeed in producing portal hypertension in dogs by ligation of the portal vein. Finally it is noteworthy that the original observations of Lebedinskaya (1932) and those of Gerez & Weiss (1936) were made in Eck fistula dogs, in which the portal venous pressure cannot be much higher than that in the inferior vena cava. For these reasons it seems justifiable to conclude that portal hypertension is not a causative factor of significance in the results obtained.

The gastric secretory stimulant which has been shown to be released by vagal excitation does not appear to increase pepsin secretion, but is obviously a potent stimulus for the parietal cell. It thus resembles both histamine and the pyloric hormone. Large amounts of histamine are present in all regions of the gastro-intestinal mucosa; gastrin is probably confined to the antral mucosa in the stomach, but appears to be present to a small extent also in the duodenal mucosa (Komarov, 1938, 1942; Uvnäs, 1942, 1943; Harper, 1946).

The fact that distension of the pyloric antrum of the stomach with a balloon excites secretion from the pouch argues that the stimulant released on feeding is, at least in part, derived from this source and that it may therefore be the gastric hormone. However, an observation so far made on one dog only suggests that the substance released by feeding is not derived exclusively from the pyloric region. At the outset of this work, when the general nature of the effects of portal venous ligation had become apparent from the results of the initial experiments, a dog was available which had a Heidenhain pouch, and in which the pyloric antrum had been removed several months before. The response of the pouch to a meal was very small. The portal vein was ligated with cellophane tape, although not as closely as later became customary. After the operation there occurred a gradual change in the pattern of response to a meal; the secretion became far greater than before operation and commenced immediately; over the same period of time the maximal secretory response to histamine greatly increased (Fig. 9). The slow onset of these changes is probably attributable to the comparative looseness with which the portal venous ligature was tied, so that diversion of the blood took place gradually. This result must obviously be accepted with considerable reserve until confirmed; but if it is accepted it indicates that the pyloric region is not the sole site of origin of the gastric secretory stimulant. It also introduces the possibility that this agent is of compound nature, i.e. more than one substance having a stimulating effect on gastric secretion is being released into the portal blood stream in the circumstances of the present experiments.

Clear evidence is afforded by the results described that the accumulation of acid in the stomach and duodenum inhibits the response of the denervated pouch which otherwise occurs on vagal excitation (teasing, sham-feeding) or distension of the pyloric antrum. The simplest interpretation of this result is that acid in the stomach and duodenum inhibits release of the gastric secretory stimulant, presumably by a nervous reflex mechanism. An alternative explanation—that the acid releases from stomach or duodenum an inhibitor of secretion—has little evidence to support it; for Code & Watkinson (1955) showed that large amounts of acid instilled into the duodenum of Heidenhain pouch dogs did not inhibit the response to histamine; and the work of Longhi, Greenlee, Bravo, Guerrero & Dragstedt (1957) appears to exclude the possibility that acid in the pyloric antrum may release an inhibitor of gastric secretion into the circulation.

In view of the evidence from these experiments that a stimulant of gastric secretion is normally released into the portal blood stream immediately food is eaten, the fact that before portal venous ligation the denervated pouch shows little or no immediate response to feeding (Fig. 1) requires further consideration; for at first sight it would seem to follow from this that the gastric secretory stimulant in question, whatever its nature, does not normally survive passage through the liver and thus enter the systemic circulation, so that it can hardly be regarded as a gastric hormone in the usual meaning of the term. Such an interpretation, however, involves the assumption that a vagally-denervated pouch is as sensitive to this substance as is the intact stomach or a vagally-innervated (Pavlov) pouch. If, in fact, the denervated pouch were relatively insensitive to the humoral stimulant in question, its response to the accumulation of this substance in the circulation after a meal would be delayed and small. The evidence of Lim & Mozer (1951) would appear to support this view, although only an abstract of their experiments has apparently been published. It seems clear that further work is required before an adequate explanation can be provided for the failure of the denervated pouch to respond immediately on feeding before portal venous ligation in the present experiments.

The magnitude of the pouch responses after portal venous ligation must be attributed largely to the general increase in sensitivity which occurs during the 3–4 weeks following the operation, as indicated by the increase in the maximal secretory response to histamine during this period; but it must not be overlooked that immediate responses of the pouches to feeding, and also responses to psychic stimulation (teasing) were obtained during the first few days after the operation of portal venous ligation, before any increase in the sensitivity of the pouch to histamine had occurred.

The cause of the striking increase in the maximal secretory response to histamine which occurs after portal venous ligation is uncertain. Whether it is associated with hyperplasia of the gastric glands has not been ascertained.

The effects on gastric secretion of the diversion of blood from portal to systemic circulations are of increasing clinical interest in view of the surgical treatment of portal hypertension by porto-caval anastomosis. Clarke, Hart & Ozeran (1958) have recently shown that in dogs provided with Heidenhain gastric pouches diversion of portal venous blood into the inferior vena cava was followed by a great increase in the secretory response to meals, the mean increase in acid production being 410% compared with controls. Diversion of portal venous blood was accomplished by the ingenious operation of porto-caval transposition, the portal vein and inferior vena cava being transected and cross-united so that portal venous blood drained into the vena cava and caval blood passed to the liver. This operation, by preserving venous inflow to the liver, is followed by little alteration in hepatic function (Silen, Mawdaley, Weirich & Harper, 1957). Clarke *et al.* (1958) also refer to their own experience and that of others with the operation of porto-caval anastomosis for the relief of portal hypertension in humans, which indicates that peptic ulceration is initiated or exacerbated following this operation. As yet, however, there appears to be little or no information concerning gastric secretory function in such patients or in those with cirrhosis in whom a portal systemic collateral circulation has developed.

SUMMARY

1. Gastric secretory responses were studied in dogs provided with gastric cannulae and completely denervated gastric pouches, before and after the diversion of portal venous blood into the systemic circulation by gradual occlusion of the portal vein.

2. This operation resulted in a great increase of the acid and volume outputs by the pouch in response to a meal. The output of pepsin was not increased. Secretion began within 10 min of feeding, reached a maximum 20–30 min later, and was largely completed in 1½ hr after feeding.

3. Teasing with food or sham-feeding caused an immediate and considerable secretion from both the main stomach and the pouch. Both responses persisted if the gastric cannula was left open. When it was closed, the response of the pouch soon ceased or declined to a low level.

4. Distension with a balloon of the antral region of the main stomach caused a persistent response from it and from the pouch if the gastric cannula was open. When it was closed, the response of the pouch soon declined to a low level.

5. The maximal secretory response of the pouch to histamine increased after portal venous ligation to at least twice the pre-operative level. This increase was first detected 5 days after portal venous ligation and attained a maximum 3–4 weeks later. It appeared to be permanent thereafter.

6. The immediate secretory responses to meals and to psychic stimulation (teasing with food) which were observed were obtained immediately after (24 hr) the operation of portal venous ligation, before an increased maximal secretory response to histamine was detectable. These responses increased in magnitude during the subsequent 3–4 weeks.

7. In one dog provided with a Heidenhain gastric pouch, and from which the pyloric antrum had been removed several months previously, portal venous ligation caused a gradual increase in the maximal secretory response to histamine and in the secretory response to a meal, similar in character to that observed in the other dogs.

8. No significant changes were observed in the gross or microscopic structure of the liver, and the animals remained in excellent health throughout the experimental period. None of them showed the phenomenon of 'meat intoxication' at any time.

9. These experiments are considered to show that a stimulant of gastric secretion is released into the portal blood stream by vagal excitation, that its release is inhibited by accumulation of acid in the stomach or duodenum, and that it is, at least in part, of gastric antral origin.

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THE EFFECTS OF METHYLPENTYNOL ON ETHANOL DRINKING AND ON WATER METABOLISM IN RATS

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According to Richter (1957) the ability of rats to make dietary selections depends on the sense of taste more than on the effects produced by substances after ingestion. As methylpentynol apparently dulls the sense of discrimination of rats (Halpern & Lehmann, 1957; Watson, 1958) it was thought of interest to see whether the administration of the drug to rats might change the distaste which they usually have for comparatively concentrated solutions of ethanol and induce them to drink solutions which they normally refuse. This in turn has led to study of the effect of methylpentynol on the water metabolism of rats. A preliminary account of this investigation has been communicated to the Physiological Society (Dicker, 1958).

METHODS

Rats of similar weight (240–285 g) and age (5–6 months) were kept either singly or in pairs in metabolism cages at 21° C. Food and fluid intake, urine and faeces excretion were measured and the animals were weighed daily. The diet was the same as that described previously (Dicker & Nunn, 1957; its calorie yield was 400 cal/100 g. The food total water content (metabolic water + moisture) was 65 ml./100 g. The average water content of faeces was 65 ml./100 g. Extrarenal water loss was estimated as the difference between total water intake (= water drunk + metabolic water + food moisture) and total water excretion (= urine + water content of faeces). Water was offered from two similar bottles, one of which might alternatively contain an ethanol solution. To avoid the effects of habituation the position of the bottles in the cages was interchanged every day (Gillespie & Lucas, 1958).

The release of pressor or oxytocic activity from the neurohypophysis was investigated, using rats of either sex anaesthetized with urethane (0·7 ml./100 g of a 25% urethane solution injected subcutaneously). The blood pressure was recorded with a mercury manometer from the femoral artery of male rats injected with 1·0 mg dibenamine (Dekanski, 1952). Uterine contractions were recorded *in situ* using a Cushny (1897) myocardiograph fixed on the horn of the uterus of rats given stilboestrol (0·01 mg/100 g) by intramuscular injection on the previous day. In both preparations one common carotid artery and a tail vein were cannulated for injections.

Drugs: for injection a solution of 3-methyl-pentyne-ol-3 (= methylpentynol) and for oral administration a solution of methylpentynol carbamate dissolved in tepid water were administered. Pitressin (Parke, Davis and Co.), batch LT488H, and Pitocin (Parke, Davis and Co.), batch LU887K, were used as vasopressin and oxytocin solutions respectively.

RESULTS

The present experiments were carried out on twenty-four rats during the winter. This may be the reason why the food (8·8 ± 0·75 g/100 g/24 hr) and the water (11·2 ± 0·17 ml./100 g/24 hr) intake were smaller than that reported for rats observed over a period of a year (Dicker & Nunn, 1957).

Intake by rats of ethanol solutions of various concentrations

When a solution of 4 % (v/v) ethanol was substituted for water in one of the two drinking bottles (Fig. 1, days 6–8), rats showed a marked preference for ethanol, which they drank to the practical exclusion of water (Richter, 1957). Total fluid intake was increased and there was a rise in urine excretion

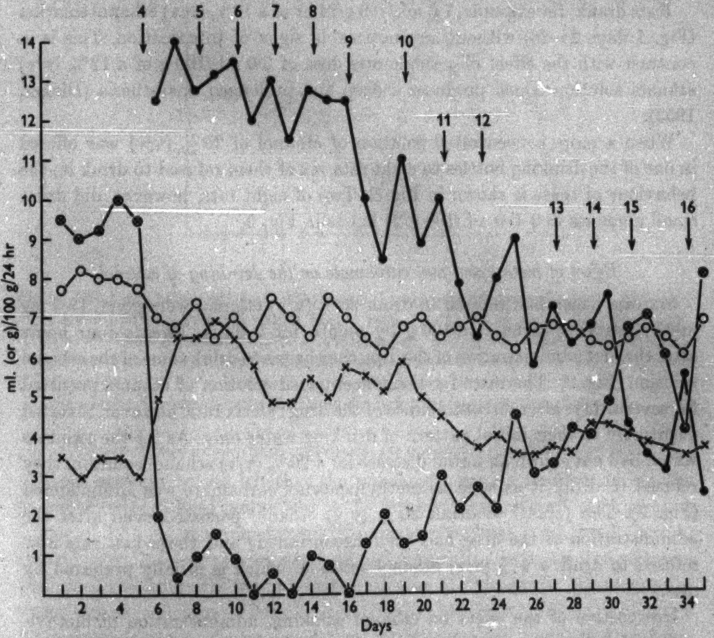

Fig. 1. Water, ethanol and food consumption in rats. ⊗—⊗, Water drunk (ml./100 g/24 hr); ○—○, food eaten (g/100 g/24 hr); ×—×, urine excretion (ml./100 g/24 hr); ●—●, ethanol solutions drunk (ml./100 g/24 hr). The numerals above the arrows indicate the concentration of the ethanol solution offered on that and following days. The results are averages obtained from observations on three pairs of rats.

(Eggleton, 1942; van Dyke & Ames, 1951) from 3·5 ± 0·48 (6) to 6·5 ± 0·82 (6) ml./100 g/24 hr (Fig. 1). The total calorie intake was 35·2 ± 1·22/100 g/24 hr; it remained unaffected by the drinking of ethanol, the animals reducing their food consumption in proportion to the intake of calories from ethanol. The calories from ethanol amounted to 4·1 ± 0·89/100 g/24 hr.

There was no appreciable change in the general metabolism when the concentration of ethanol was increased by 1% (v/v) at a time until it reached 9% (v/v). Above that concentration the volume of ethanol solution drunk decreased (Fig. 1), though the mean calorie intake from ethanol remained unchanged at 3·9 ± 0·76 cal/100 g/24 hr. The deficit of liquid resulting from the decreased consumption of ethanol solutions was made good by an increase of water intake. Preference for ethanol disappeared if its concentration exceeded 15% (v/v) (Fig. 1).

Rats drank, for example, 7·0 ml./100 g/24 hr of a 12% (v/v) ethanol solution (Fig. 1 days 24–26) without any noticeable signs of intoxication. This is in contrast with the effect of a single oral dose of 5·0 ml./100 g of a 12% (v/v) ethanol solution which produces a deep and prolonged anaesthesia (Dicker, 1953).

When a more concentrated solution of ethanol of 20% (v/v) was offered in one of the drinking bottles to eight rats, six of them refused to drink it; the behaviour of these is shown in Fig. 2. Two of eight rats, however, did drink small amounts (3·0–5·0 ml./100 g/24 hr) as in Fig. 3.

Effect of methylpentynol carbamate on the drinking of ethanol

Six rats which had refused to drink 20% (v/v) ethanol were given 12·5 mg methylpentynol carbamate/100 g by mouth for 5 days. Twenty-four hours after the first administration of the drug they began to drink some of the ethanol solution (Fig. 2). The taste for the concentrated solution of ethanol persisted for several days after discontinuance of the drug; all six rats, however, reverted eventually to their initial pattern of drinking water only. As for the two rats which had not shown an initial distaste for a 20% (v/v) ethanol solution, they refused to drink it as soon as methylpentynol carbamate was administered (Fig. 3). The refusal to drink 20% (v/v) ethanol persisted even after the administration of the drug had been discontinued; and these two rats now refused to drink a 4% (v/v) ethanol solution, which is usually preferred by rats.

Irrespective of the effect on ethanol drinking, administration of methylpentynol carbamate (12·5 mg/100 g) decreased the fluid intake in all rats (Figs. 2, 3) without a concurrent decrease of urine excretion.

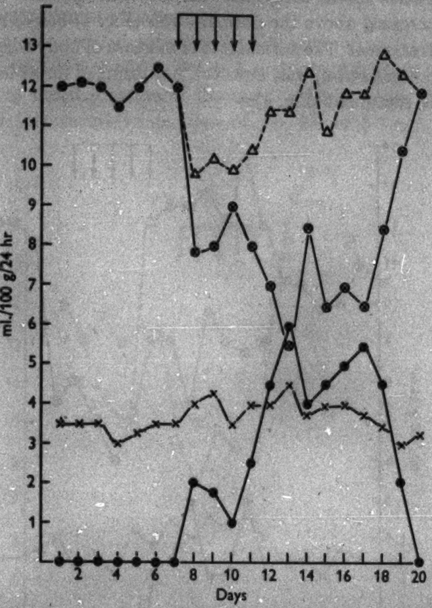

Fig. 2. Effect of administration of methylpentynol carbamate on the drinking of a 20% (v/v) ethanol solution; averaged results from 6 rats. ⊗—⊗, water drunk (ml./100 g/24 hr); ×—×, urine excretion (ml./100 g/24 hr); ●—●, 20% (v/v) ethanol solution drunk (ml./100 g/24 hr); △---△, total liquid (water + ethanol solution) intake (ml./100 g/24 hr). Arrows indicate the successive doses of methylpentynol carbamate (12·5 mg/100 g) administered. Note the decrease of fluid intake during the administration of the drug.

Effects of methylpentynol carbamate on the water metabolism of rats

In an attempt to see whether the unchanged urine excretion during the period of reduced fluid intake was partly due to the diuretic effect of ethanol, a dose of 12·5 mg methylpentynol carbamate/100 g was given for 5 days to control rats allowed free access to food and water. In all six rats, the administration of the drug resulted in a marked degree of hypodipsia; the amount of water drunk fell from 11·5 ± 0·75 to 8·3 ± 0·56 ml./100 g/24 hr without a concurrent decrease of urine excretion, which remained unaffected at 3·8 ± 0·62 ml./100 g/24 h. Food consumption remained at 9·1 ± 0·21 g/100 g/24 hr, but the body weight fell sharply (Fig. 4). Minimizing the progressive dehydration, extrarenal water loss decreased from 12·6 ± 0·61 to 7·4 + 0·5 ml./100 g/24 hr, and the water content of faeces was reduced from 65 to 45%. After the administration of the drug had been discontinued, the volume of water drunk first increased above the normal levels (Fig. 4) before returning to the value before treatment. The normal rate of increase of body weight (0·8 g/100 g/24 hr in the present series) was, however, not resumed for at least 7 days after the end of the treatment (Fig. 4).

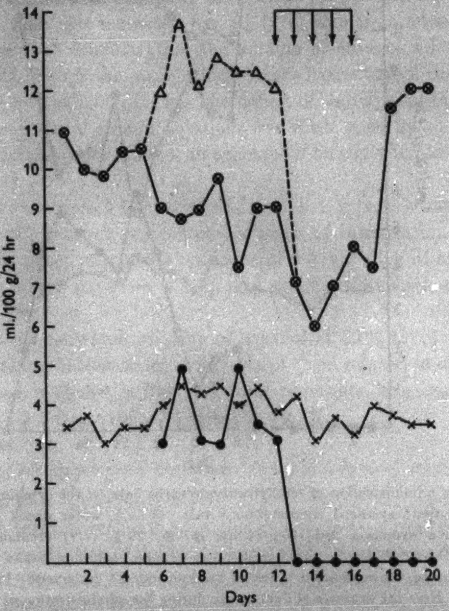

Fig. 3. Effect of methylpentynol carbamate on rats which drank a 20% (v/v) ethanol solution. The results are the averages from two rats. Symbols as for Fig. 2.

Effects of methylpentynol on the release of posterior pituitary hormones

It has been shown previously that injections of hypertonic sodium chloride solutions into the carotid artery produce an antidiuretic (Friedman, Hinke & Friedman, 1956) and a pressor effect in the rat (Dicker & Nunn, 1958), and a release of both oxytocic and antidiuretic activities in the dog (Abrahams & Pickford, 1954). The release of these hormones following the intracarotid injection of hypertonic saline solution can be inhibited by ethanol (van Dyke & Ames, 1951; Dicker, 1954). As methylpentynol belongs to the group of alcohols, it was of interest to see whether the observed dissociation between hypodipsia and urine excretion could be explained by an inhibition of the release of posterior pituitary hormones.

Anaesthetized rats, treated with dibenamine, received an injection through the common carotid artery of 0·1, 0·2 or 0·3 ml. of a 3% NaCl solution. This produced rises of blood pressure of the same order as those observed after intravenous (or intracarotid) injections of 1·0, 2·0 or 3·0 m-u. vasopressin, respectively (Dicker & Nunn, 1958). After an intracarotid injection of methylpentynol (5 mg/100 g), there was no pressor response to osmotic stimulation though the response to injection of vasopressin remained unchanged (Fig. 5).

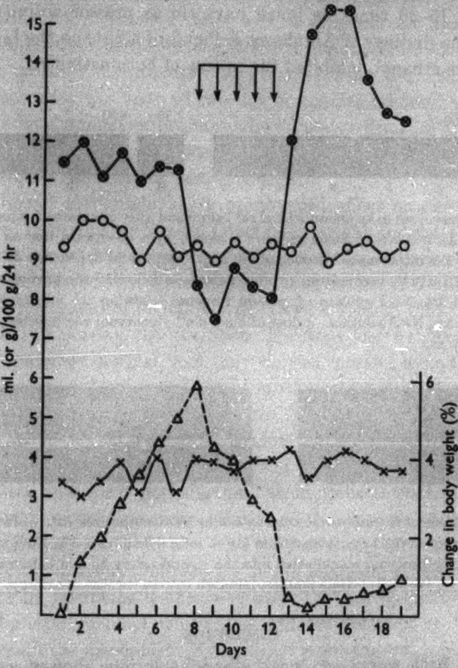

Fig. 4. Effects of methylpentynol carbamate on body weight and water metabolism of six control rats. ⊗—⊗, water drunk (ml./100 g/24 hr); ○—○, food eaten (g/100 g/24 hr); ×—×, urine excretion (ml./100 g/24 hr); △---△, increase in body weight expressed as percentage. Arrows indicate daily administration of methylpentynol carbamate (12·5 mg/100 g).

In anaesthetized female rats, intracarotid injection of 0·3 ml. of a 3% NaCl solution produced contraction of the uterus which could be matched by that produced by intravenous injections of 45–60 m-u. oxytocin, as in the example of Fig. 6. After intracarotid injection of methylpentynol (5 mg/100 g) uterine responses to osmotic stimulation of the neurohypophysis disappeared entirely, though the uterus continued to retract normally to further injections of oxytocin.

Here and in the earlier experiments of Dicker & Nunn (1958) the intracarotid injection of 0·3 ml. of a 3 % NaCl solution released from the posterior pituitary gland about 15–20 times as much oxytocic as pressor activity in the rat, confirming the findings of Abrahams & Pickford (1954) on the bitch; methylpentynol, like ethanol, inhibited the release of both activities.

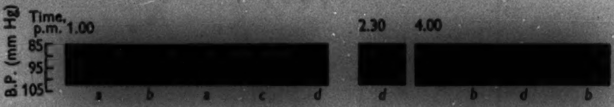

Fig. 5. Pressor responses in an anaesthetized rat before and after intracarotid injection of methylpentynol. An increase of blood pressure is shown by a downward line on the tracing. *a*, response to the intravenous injection of 2·0 m-u. vasopressin; *b*, response to the intravenous injection of 1·0 m-u. vasopressin; *c*, pressor response following the intracarotid injection of 0·1 ml. of a 3 % NaCl solution; *d*, pressor response following the intracarotid injection of 0·2 ml. of a 3 % NaCl solution. A dose of 5 mg methylpentynol was injected into the carotid artery at 2 p.m.

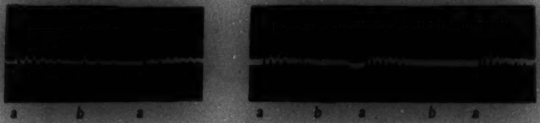

Fig. 6. Contractions of the uterus recorded *in situ* in an anaesthetized rat. *a*, Intravenous injection of 60 m-u. oxytocin; *b*, intracarotid injection of 0·3 ml. of a 3 % NaCl solution. A dose of 5 mg methylpentynol was injected into the carotid artery 30 min before the beginning of the tracing on the right. Time marker, minutes.

DISCUSSION

Rats offered both water and weak ethanol solutions showed a marked preference for the latter, which they drank to the practical exclusion of water (Richter, 1957). When the concentration of ethanol was increased above 15 % (v/v), preference for ethanol disappeared in the great majority of cases. Two out of eight rats, however, drank small amounts of 20 % (v/v) ethanol solution. It is interesting to note that though all the rats used in this investigation were approximately of the same age, these two animals were 90 and 105 g heavier than the average weight of the colony. Richter (1957) has pointed to the possible relation between basal metabolism and 'alcohol craving' in rats.

Little is known about the mode of action of methylpentynol. It is neither an analgesic nor an anaesthetic drug (Margolin, Perlman, Villani & McGavack, 1951); it has, however, a definite hypnotic and sedative effect (Margolin *et al.* 1951; Halpern, 1956) which, according to Halpern & Lehmann (1956), results from its action on the hypothalamus and on the reticular formation of the brain. Given to rats, it increases both their activity and their exploratory behaviour (Dicker, Steinberg & Watson, 1957) but decreases their ability to learn (Watson, 1958). In men faced with a difficult task methylpentynol appears to impair their performance (Dicker & Steinberg, 1957).

From the present investigation it would appear that methylpentynol carbamate can alter the taste of rats for or against ethanol: under the influence of the drug, animals which normally refused to drink 20% (v/v) ethanol solution drink it, and those few rats which drank such concentrated solutions refused to drink ethanol in any concentration.

Methylpentynol carbamate has a pronounced effect on the water metabolism of rats: it produces a marked fall of body weight and a noticeable hypodipsia without concurrent decrease of urine excretion. This is reminiscent of the syndrome observed in dogs after electrocoagulation of the lateral part of the anterior hypothalamus (Witt, Keller, Batsel & Lynch, 1952; Andersson & McCann, 1955): following the operation, the dogs lost weight though their food intake remained normal, and they showed marked hypodipsia, though the urine excretion did not appear to be much decreased. Indeed, Witt *et al.* (1952) suggested the injection of Pituitrin (pituitary extract; Parke, Davis and Co.) as a requisite therapy against the loss of fluid through urine excretion. It is therefore possible that one of the sites of action of methylpentynol lies in the region of the anterior hypothalamus. This hypothesis would agree not only with the 'autonomous depressant effects' of the drug as observed in man (Dicker & Steinberg, 1957) but also with the fact that, like ethanol, it inhibits the release of the neurohypophysial hormones following osmotic stimulation. This may not be surprising if one remembers that methylpentynol is an unsaturated aliphatic carbinol which, like ethanol, belongs to the group of alcohols. Though the mechanism of inhibition is not known, it is possible that both drugs interfere in the hypothalamus with the release of acetylcholine in a way similar to that observed for methylpentynol in a frog nerve-muscle preparation (Nicholls & Quilliam, 1956). If this is so, the difference between their actions would be narrowed to that of a greater potency of methylpentynol. The fact that, in contrast with ethanol, methylpentynol is not attacked by alcohol-dehydrogenase (A. L. Greenbaum, personal communication) may account for this difference.

SUMMARY

1. Rats given a choice of water or ethanol solutions to drink showed a clear preference for the latter, as long as the concentration of ethanol did not exceed 15% (v/v).

2. When both a 20% (v/v) ethanol solution and water were offered, the majority of rats drank water only. Under the influence of methylpentynol carbamate, however, they drank some of the 20% (v/v) ethanol solution.

3. Administration of methylpentynol carbamate (12·5 mg/100 g/day for 5 days) to control rats kept in metabolism cages with free access to food and water produced a marked hypodipsia without concurrent decrease of urine flow. The food intake remained unchanged, but the body weight decreased appreciably.

4. Intracarotid injection of 5 mg methylpentynol in anaesthetized rats prevented the release of both pressor and oxytocic activities in response to osmotic stimulation of the neurohypophysis. It is suggested that one of the possible sites of action of methylpentynol lies in the anterior part of the hypothalamus.

I wish to thank Miss Joan Nunn for her technical skill and her constant help.

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THE EFFECTS OF STIMULATION OF THE CAROTID BODY CHEMORECEPTORS ON HEART RATE IN THE DOG

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It is well known that hypoxia causes tachycardia, but there is no general agreement as to the mechanism of this effect. Some workers have assumed it to be the result of a reflex arising from stimulation of the carotid body and aortic arch chemoreceptors (Asmussen & Chiodi, 1941; von Euler & Liljestrand, 1942; Whitehorn, Edelmann & Hitchcock, 1946; Dripps & Comroe, 1947; Alveryd & Brody, 1948), despite there being a number of conflicting reports of the effects of stimulation of the chemoreceptors on heart rate. Stimulation of the carotid bodies by various drugs injected into the common carotid artery of spontaneously breathing dogs caused bradycardia (Heymans, Bouckaert & Dautrebande, 1931*a*, *b*; Heymans, Bouckaert, von Euler & Dautrebande, 1932; Heymans, Bouckaert, Farber & Hsu, 1936; Comroe & Schmidt, 1938; Heymans & Bouckaert, 1941), but perfusion of the carotid sinus region using Ringer's solution with either a high CO_2 content or a low pH resulted in tachycardia (Heymans, Bouckaert & Dautrebande, 1930; Heymans, Bouckaert & Samaan, 1935). More recently, Bernthal, Greene & Revzin (1951) excited the carotid bodies by hypoxic blood and found variable effects on heart rate. In every case chemoreceptor stimulation, whether by drugs, Ringer's solution or hypoxic blood, caused reflex hyperpnoea. On the other hand, in dogs in which the rate and depth of respiration were controlled by a pump, stimulation of the carotid bodies by either hypoxic or venous blood invariably caused bradycardia (Bernthal *et al.* 1951; Daly & Daly, 1957).

In the cat Landgren & Neil (1952) found that stimulation of chemoreceptors by local application of various drugs to the carotid bodies invariably caused hyperpnoea, hypertension and tachycardia. In subsequent experiments, in which carotid body perfusion techniques were used, Neil (1956) showed that the tachycardia which occurs in systemic hypoxia was not the result of stimulation of the carotid body chemoreceptors.

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In the course of some experiments, we had observed, as had Bernthal *et al.* (1951), that chemoreceptor stimulation caused variable effects on heart rate in dogs spontaneously breathing room air. The present study was therefore undertaken with a view to obtaining further information on the mechanisms responsible for these responses, in the hope that it might throw some light on the cause of the tachycardia occurring in hypoxia. Some of our results have been described briefly (Daly & Scott, 1958).

METHODS

Dogs varying in weight from 10·3 to 24·6 kg were premedicated with morphine hydrochloride (1–2 mg/kg subcutaneously). Half an hour later they were anaesthetized with a mixture of chloralose (0·05 g) and urethane (0·5 g/kg intravenously). Systemic blood pressure was measured from a femoral artery by means of a Hürthle manometer. Heart rate was counted from the blood pressure record taken on a fast moving paper or was recorded on the kymograph by a Gaddum drop timer (Gaddum & Kwiatkowski, 1938) using the method by Daly & Schweitzer (1950).

The animals breathed room air through valves of low resistance. Respiratory minute volume was measured in one of three ways: (1) by collection of expired air in a balanced spirometer for a given period of time; (2) by passing the expired air through a gas meter, every 0·5 l. expired air being signalled on the kymograph. When either of these methods was used intratracheal pressure was also recorded by means of a damped Marey tambour as a qualitative measure of the changes in respiration. Tidal air volume was calculated from values for the respiratory minute volume and the respiratory rate, the latter being obtained from the intratracheal pressure trace. (3) The third way was by measurement of tidal air volume recorded by means of a small balanced spirometer connected to a conventional closed-circuit respiratory system. Carbon dioxide was absorbed by two soda-lime towers and the flow of oxygen into the system was controlled by an adjustable needle valve so as to maintain the lower (expiratory) limiting line of the tidal air tracing horizontal. Respiratory minute volume was calculated as the product of the tidal volume and the ventilatory rate.

When necessary, artificial positive pressure ventilation was applied by means of a Starling 'Ideal' pump. In some experiments, a bilateral open pneumothorax was created and in these the lungs collapsed passively against a resistance of 2–3 cm H_2O.

In two experiments the lungs were ventilated by an intermittent negative pressure applied to the thorax only, the principle being similar to that employed by Sauerbruck (1904). During preparation of the animal positive pressure artificial ventilation was applied. The chest was opened by splitting the sternum in the mid line and the internal mammary vessels were ligated. The phrenic nerves were crushed and passive movements of the diaphragm caused by changes in intrathoracic pressure were prevented by a metal plate placed above the diaphragm and fixed to the ribs. A specially shaped Perspex plate, maximum width 10 cm, was inserted under the sternum so as to seal the thorax again. The edges of the plate against the ribs were made air-tight by the liberal use of soft paraffin. A tube attached to the centre of the plate was connected to a negative pressure chamber from which air was continually exhausted by means of an electric vacuum pump. Air was admitted to the chamber by a cam-operated valve 18 times/min. The pressure fluctuations in the chamber were thus transmitted to the animal's thorax. The intrathoracic pressure during the expiratory phase of respiration was -2 cm H_2O. When the necessary connexions had been made, negative pressure ventilation was substituted for positive pressure ventilation and the tidal air volume was measured by means of a small balanced spirometer connected to a conventional closed-circuit respiratory system.

Perfusion of the carotid bodies

Reflex effects from the carotid body chemoreceptors were elicited by temporarily changing the perfusate from oxygenated to hypoxic blood. The hypoxic blood was obtained from a donor dog breathing 5 or 7% O_2 in N_2. Both carotid bifurcation regions were isolated from the circulation by ligation of all branches of the common and external carotid arteries. The veins draining the carotid bodies were preserved (Chungcharoen, Daly & Schweitzer, 1952). The arrangement of the perfusion apparatus is shown diagrammatically in Fig. 1. The carotid sinuses and bodies were perfused by means of a Bayliss–Müller (1928) type of roller pump. The input side of the pump was connected via the three-way tap *a* to the central end of a carotid artery of the recipient animal for perfusion of the carotid bodies with oxygenated blood, and to the central end of a femoral artery of the donor animal for perfusion with hypoxic blood. The blood perfusing both carotid sinus regions was returned, via the cannulated external carotid arteries, a Starling type of resistance *b* and a second three-way tap *c*, to a femoral vein of either the recipient or the donor animal. The direction of the taps was such that the blood was always returned to the animal from which it came. A warming coil, at 37° C, was placed between the pump and the common carotid cannulae so that the temperature of the blood perfusing the carotid bodies did not vary by more than 0·5° C on changing the perfusate. The output of the pump was approximately 100 ml./min and the dead space between tap *a* and the common carotid cannulae was about 10 ml.

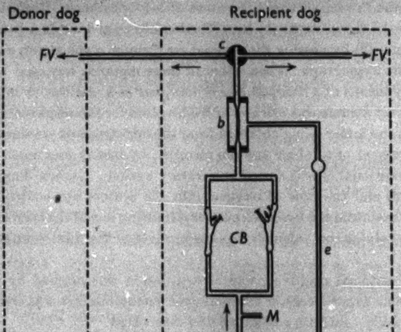

Fig. 1. Diagram showing the method of perfusion of the carotid bodies with either oxygenated blood from the recipient or hypoxic blood from the donor dog. Both carotid bifurcation regions are perfused through the common carotid arteries by means of a Bayliss–Müller (1928) type of roller pump with carotid arterial blood from the recipient animal via the three-way tap *a*. The third limb of the tap *a* is connected to a femoral artery of the donor dog. Blood leaving the carotid sinuses by the external carotid arteries is returned to a femoral vein of the recipient animal via the Starling resistance *b* and three-way tap *c*. The third limb of this tap is connected to a femoral vein of the donor dog. The Starling resistance is connected either to the recipient's carotid artery via tube *e* (as shown) or to a pressure bottle. Side tube *d* is temporarily opened to fill tube from donor with a representative sample of blood. *CA*, from carotid artery; *CB*, carotid bifurcation region; *FA*, from femoral artery; *FV*, to femoral vein; *M*, to manometer; for further details see text.

In some experiments the pressure in the carotid sinuses, measured with a mercury manometer, was maintained at the same level as that in the systemic arteries. This was achieved by connecting the Starling resistance directly to a carotid artery of the recipient animal (tube *e* in Fig. 1). Thus changes in systemic blood pressure caused similar changes in pressure not only in the Starling resistance but also in the carotid sinuses, because the blood flow through the resistance remained constant. In other experiments the carotid sinus pressure was maintained constant by connecting the Starling resistance to a pressure bottle.

The donor dog either spontaneously breathed room air through a respiratory valve or was ventilated artificially. The gas mixture, 5 or 7% O_2 in N_2, was administered from a Douglas bag. In practice, the low oxygen mixture was given for a period of about 2 min and then a clamp on the side-tube *d* (Fig. 1) was opened to fill the tube connecting the femoral artery of the donor to tap *a* with a representative sample of blood. Finally, the carotid bodies of the recipient animal were stimulated temporarily by turning taps *a* and *c* through 180°. When the test was over, room air was substituted for the gas mixture in the donor dog.

Both the donor and recipient animals were given heparin (Liquemin, Roche Products Ltd. 21–24 mg/kg) to render the blood incoagulable.

Perfusion of the brain

In order to delay the arrival of blood from the lungs at the brain, the brain of the recipient dog was perfused through the vertebral arteries by a Dale–Schuster pump with blood from the central end of a carotid artery. About 9 m of polythene tubing, 6 mm bore, connected the output side of the pump with the cannulae in the vertebral arteries. Thus taking into account the dead space in the pump and tubing and the rate of blood flow, the arrival of blood at the brain was delayed by about 2 min. The polythene tubing was immersed in a water-bath maintained at a temperature of 37° C. To obviate the possibility that blood from the systemic circulation might reach the brain directly through the anterior spinal arteries, the output of the pump was adjusted so that the cerebral perfusion pressure was maintained about 50 mm Hg higher than the systemic blood pressure. No carotid blood reached the brain because in these experiments the carotid bifurcation regions were isolated from the circulation and perfused as described above.

Preparation of animals for subsequent denervation of the lungs

In some animals the lungs were denervated during the acute experiment without having to open the thorax. A preliminary operation was performed under sodium pentobarbitone (Nembutal, Abbott Laboratories, Ltd.) anaesthesia with full aseptic precautions. The vagosympathetic nerve below the hilum of the lung was divided on each side, and at the same time a snare was placed on the vagosympathetic nerves immediately above the hilum but below the origin of the cardiac branches of the vagus. Thus when these nerves were severed at the final experiment performed 4–14 days later, only the lungs were denervated.

Each snare consisted of a loop of stainless steel wire, 0·14 mm diameter, running in a guide of polythene tubing. On the left side the loop of wire was placed round the nerve at the level of the inferior border of the aortic arch and on the right side at the level of the azygos vein. The vein was divided between ligatures to facilitate exposure of the nerve. The polythene tubes passed through the fourth intercostal space on either side, and their ends, together with the protruding lengths of wire, were buried beneath the skin. These tubes were subsequently exposed through a skin incision at the time of the final experiment. Each nerve was severed by holding the polythene tube firmly and pulling sharply on the ends of the wire. Since nerve fibres to the lungs may reach their destination by pathways other than the thoracic vagosympathetic nerves, we cannot be certain that complete denervation of the lungs is effected by this method. Our results indicate, however, that the majority of the fibres are divided.

Control experiments indicated that denervation of the lungs in this way did not interrupt the vagal efferent nerve supply to the heart to any appreciable extent.

RESULTS

Effects of stimulation of the carotid body chemoreceptors

Dogs artificially ventilated

It was found in the artificially ventilated animal with open or closed chest that stimulation of the carotid bodies by hypoxic blood invariably caused a reduction in heart rate. The typical effect is shown in Fig. 2. It may be noted that there is a delay of about 15 sec in the cardiac responses to changes in the carotid body perfusate from oxygenated to hypoxic blood and later from hypoxic to oxygenated blood. This is due largely to the dead space in the perfusion system.

Fig. 2. Dog, ♂, 16·2 kg. Morphine-chloralose-urethane; closed chest; positive pressure ventilation. Carotid sinus perfusion pressure maintained constant. Separate perfusion of cerebral circulation through the vertebral arteries. Decamethonium iodide 5 mg before recording began. During signal the carotid bodies were stimulated by hypoxic blood. The figures below the blood pressure trace are those for heart rate (beats/min). In this and in subsequent figures: T.A. = tidal air volume (inspiration upwards); R.M.V. = respiratory minute volume; Resp. = intratracheal pressure; C.S.P. = carotid sinus pressure; B.P. = systemic blood pressure; H.R. = heart rate. Time marker, 10 sec.

The results, which are summarized in Table 1, show that the average reduction in heart rate in four open-chest preparations was 40·7 % (range 13–57 %; nine observations) and in seven closed-chest preparations was 38·3 % (range 10·3–85 %; eleven observations). In six out of seven experiments carried out on preparations with closed chest, it was necessary to give decamethonium iodide (Eulissin 'A', Allen and Hanburys, Ltd.) to prevent violent spontaneous respiratory efforts against the pump occurring during chemoreceptor stimulation. It is unlikely that decamethonium itself influenced the heart rate responses to carotid body stimulation, because in open-chest preparations the response was the same before and after giving the drug. Furthermore, in one closed-chest preparation in which spontaneous respiratory efforts were weak and no decamethonium was administered, chemoreceptor stimulation resulted in a 10 and 21 % reduction in heart rate respectively in two tests.

The observed heart-rate responses occurred independently of the changes in systemic blood pressure, which either increased, decreased or remained unchanged. They also occurred whether the carotid sinus perfusion pressure was maintained constant or was allowed to vary with the systemic blood pressure (see Methods). The bradycardia occurring in response to chemoreceptor stimulation was reduced or abolished by atropine (three experiments) and considerably reduced by division of both cervical vagosympathetic nerves (one experiment). It was abolished by division of the carotid sinus nerves (three experiments).

TABLE 1. The effects of stimulation of the carotid bodies by hypoxic blood on heart rate in animals artificially ventilated

		Heart rate		
No. of expts.	No. of tests	Control (beats/min)	Decrease (beats/min)	Decrease (%)
		Open-chest preparations		
4	9	126–234 (177·3)	30–102 (68·7)	13–57 (40·7)
		Closed-chest preparations		
7	11	108–200 (154·1)	18–106 (69·3)	10·3–85 (38·3)

The open figures indicate the range of values, those in parentheses the mean.

In one further experiment the carotid bodies were stimulated by hypercapnic blood. For this purpose, the donor dog was given a gas mixture containing 10 % CO_2 and 21 % O_2 in N_2 to breathe. In one such test chemoreceptor stimulation caused a reduction in heart rate from 114 to 70 beats/min (39 %).

These results indicate that when the rate and depth of ventilation are controlled, thereby maintaining the arterial blood pO_2 and pCO_2 constant, or very nearly so, carotid chemoreceptor stimulation causes a primary reflex bradycardia of vagal origin.

Dogs breathing spontaneously

In thirty-two tests made on six dogs spontaneously breathing room air, chemoreceptor stimulation invariably caused an increase in respiratory minute volume varying from 29 to 570 % of the control value. The heart rate increased in sixteen tests, decreased in eight and did not change in eight (Table 2). These results were observed after a steady state had been reached, for in some tests the heart slowed before accelerating to a rate above the control value. In some experiments sinus arrhythmia was present during the control period and this became more evident during stimulation of the chemoreceptors. It was found, however, that despite the heart becoming more irregular, the change in rate counted over a period of ½–1 min was again variable.

These chronotropic effects on the heart were not dependent on the changes in systemic blood pressure and, furthermore, they occurred whether the carotid sinus pressure was maintained constant or was allowed to vary with the systemic blood pressure. The cardio-accelerator responses to carotid body stimulation occurred in the bilaterally adrenalectomized preparation. All cardiovascular and respiratory responses were abolished after division of the carotid sinus nerves.

In the above experiments the hypoxic blood used for stimulation of the carotid bodies was obtained from a donor dog spontaneously breathing 5 or 7% O_2 in N_2. Tests showed that the variable responses on heart rate were not related to accompanying reduction in the pCO_2 of the perfusate, because similar results were obtained when the carotid bodies were stimulated with hypoxic blood from a donor dog artificially ventilated with 5 or 7% O_2 in N_2.

TABLE 2. The effects of stimulation of the carotid bodies by hypoxic blood on heart rate in six dogs spontaneously breathing room air

No. of tests	Heart rate (beats/min) Control	Increase	Decrease	Change in heart rate (%)
16	93–126 (109·8)	3–30 (18·6)	—	+2·5 to +29 (+17·5)
8	84–204 (112·5)	—	3–48 (14·0)	−3·1 to −23·5 (−9·3)
8	84–220 (133·7)	—	—	0

The open figures indicate the range of values, those in parentheses the mean.

There was no consistent relationship between the initial heart rate and the direction of the response to stimulation of the chemoreceptors. As may be seen from Table 2, the average initial heart rate in the group of tests in which a tachycardia occurred was about the same as that in which bradycardia took place. On the other hand, the average initial heart rate was somewhat higher in tests in which chemoreceptor stimulation had no chronotropic effect. The absence of any change in heart rate in response to carotid body stimulation was not due to the chemoreceptors being inactive. The reasons for this conclusion are as follows. First, in all eight tests in which no change in heart rate occurred there was a reflex increase in respiratory minute volume. Secondly, in three animals subsequent tests of carotid body stimulation caused either an increase or a decrease in heart rate; and thirdly, when in the same three animals, the rate and depth of respiration were maintained constant by a pump, chemoreceptor stimulation invariably caused a bradycardia. In connexion with this last finding it was also observed that when the response to stimulation of the carotid bodies in the spontaneously breathing animal was bradycardia, it was enhanced when the test was repeated whilst the animal was ventilated artificially.

In three experiments carried out on dogs breathing spontaneously it was noted that repeated stimulations of the chemoreceptors by hypoxic blood caused not only variations in the direction of the response of the heart rate but also in the percentage increase in respiratory minute volume. There was a tendency for the increases in heart rate to be associated with the larger changes in respiratory minute volume and for the decreases in rate to go with the smaller ventilatory responses. This is evident from Fig. 3 which shows the relationship between the percentage change in heart rate and the percentage increase in respiratory minute volume for thirty-two tests carried out on six animals. The correlation coefficient between the percentage changes in heart rate and respiratory minute volume is 0·68 and between the actual changes is 0·78. Both these values are statistically highly significant ($P<0{\cdot}001$).

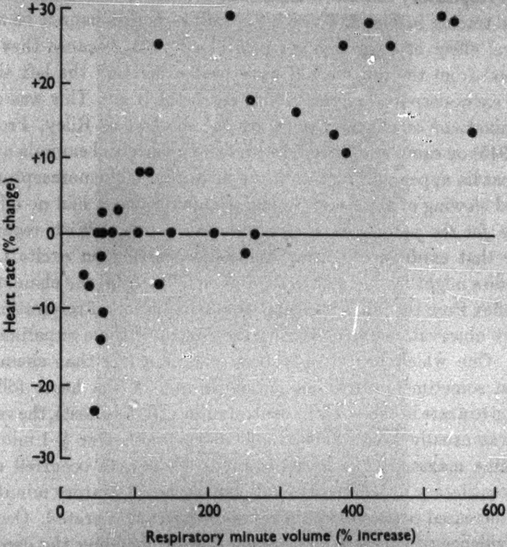

Fig. 3. The relationship between the percentage change in heart rate and percentage increase in respiratory minute volume occurring on stimulation of the carotid body chemoreceptors by hypoxic blood in dogs spontaneously breathing room air.

Since the primary reflex effect of chemoreceptor stimulation is to cause bradycardia, it is evident that some other mechanism must be responsible for the cardio-accelerator response. There would appear to be several possible mechanisms which might contribute to this response: the two to which we confined ourselves in this study were, first, a direct central effect of a reduction in cerebral blood pCO_2 caused by the reflex hyperventilation; and secondly, a reflex originating in the lungs consequent upon the increased inspiratory volume of the lungs (Hering, 1871; Anrep, Pascual & Rössler, 1936*a*, *b*) as postulated by Bernthal *et al.* (1951).

Perfusion of the brain. In three experiments in which the brain was perfused through the vertebral arteries it was found in eleven of fifteen tests that stimulation of the carotid bodies by hypoxic blood caused tachycardia and an increase in respiratory minute volume. The typical effect is shown in the first part of the tracing in Fig. 6. These heart rate responses cannot be attributed to a central effect of a lowered cerebral blood pCO_2 because they occurred before blood from the lungs could have passed through the left side of the heart and extra-corporeal circulation to reach the brain. This was confirmed by determinations of pO_2 and pCO_2 by the method of Riley, Proemmel & Franke (1945) on samples of blood drawn from a vertebral cannula at the time the tachycardia appeared. In two of the fifteen tests chemoreceptor stimulation caused slowing of the heart; in the remaining two it had no effect.

Evidence for the participation of a pulmonary reflex. With regard to the possibility that cardio-accelerator responses occurring on excitation of the carotid bodies might be due, not to a primary reflex from the chemoreceptors, but to a reflex from the lungs resulting from stimulation of respiration, certain preliminary observations made during the course of these experiments seem pertinent. One which has already been mentioned is that chemoreceptor stimulation sometimes caused an initial slowing of the heart followed by acceleration to a rate in excess of the control value. In these tests, the ventilatory response was usually slow in onset, and it was only after $\frac{1}{2}$–1 min that the effect became maximal. The initial bradycardia always occurred at a time when the ventilatory response was small, but as the respiratory minute volume gradually increased to its maximum value the heart accelerated. Occasionally the first evidence of a ventilatory response after changing the carotid body perfusate from oxygenated to hypoxic blood was a single deep breath and this was accompanied by a momentary increase in heart rate. Such an effect is shown in Fig. 4. In another experiment in which this effect was prominent the heart rate increased from its control value of 98 to 180 beats/min during the single deep breath. A compensatory apnoea followed during which the rate fell to 75 beats/min. These observations suggested that acceleration of the heart during chemoreceptor stimulation might be related to the changes in inspiratory volume of the lungs consequent upon reflex stimulation of respiration. The mechanism may be inhibition of the cardio-inhibitory centre as a result of either irradiation from the respiratory centre or a stretch reflex from the lungs (Anrep *et al.* 1936*a*, *b*).

Evidence for the participation of a pulmonary reflex was obtained from two types of experiment. In the first the heart rate responses to carotid body stimulation were observed before and after denervation of the lungs. The results of three such experiments are shown in Table 3. In two (Expts. nos. 19 and 20) the control response to chemoreceptor stimulation by hypoxic blood was a reduction in heart rate. This response was increased after denervation of the lungs. In the third experiment (no. 23 in Table 3) three of four control tests resulted in increases in heart rate of 14, 17 and 9% respectively; in the fourth, no change occurred. After lung denervation, however, chemoreceptor

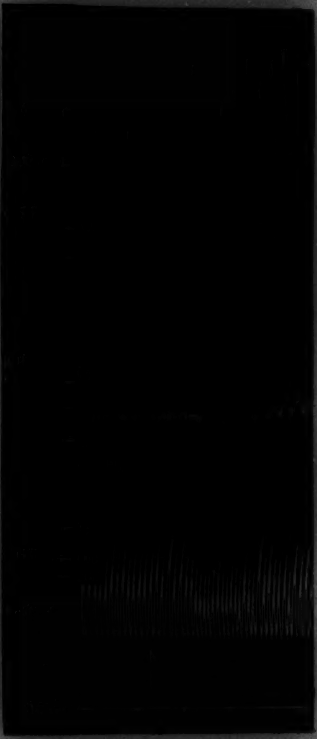

Fig. 4. Dog, ♀, 12·0 kg. Morphine-chloralose-urethane; spontaneous respiration. Carotid sinus perfusion pressure maintained at the same level as the systemic blood pressure. Tidal air volume was measured by means of a spirometer connected to a conventional closed-circuit respiratory system. Bilateral adrenalectomy. At arrow ↑ stimulation of the carotid body chemoreceptors by hypoxic blood was begun. Note the first effect on respiration is a simple deep inspiration which is accompanied by acceleration of the heart from about 155 to 200 beats/min.

stimulation caused reductions in heart rate of 17, 13 and 15% respectively in three consecutive tests. In all three experiments lung denervation had only a small effect on the ventilatory response to chemoreceptor stimulation (Table 3).

These experiments suggest that carotid body stimulation simultaneously excites two mechanisms having opposite effects on heart rate: a primary reflex bradycardia and an accelerator mechanism which is reduced or abolished by denervation of the lungs. This would account for the enhancement by lung denervation of the cardio-inhibitory responses occurring in Expts. 19 and 20, and for the reversal of the response in Expt. 23 (Table 3).

TABLE 3. The effects of stimulation of the carotid body chemoreceptors on heart rate and respiration before and after denervation of the lungs in dogs spontaneously breathing room air. *C* and *E* are the control and experimental states respectively

Expt. no.		Heart rate (beats/min)		Change (%)	Resp. minute volume (l./min)		Resp. rate (c/min)		Tidal air volume (ml.)	
		C	*E*		*C*	*E*	*C*	*E*	*C*	*E*
19	*a*	111	108	−3	2·5	10·0	16	33	156	302
	b	108	105	−3	3·3	8·0	20	28	165	285
	*c**	111	87	−21·6	2·5	9	10	18	250	500
20	*a*	132	87	−34·2	4·9	21·0	15	27	327	780
	b	102	70	−31·4	3·9	15·7	15	27	260	582
	c	102	72	−29·4	5·25	11·5	22	24	239	480
	*d**	82	24	−71	4·3	18·0	11	21	391	860
	*e**	68	34	−50	4·3	16·7	10	—	430	—
23	*a*	116	132	+14	4·28	11·2	16	32	268	350
	b	117	141	+17	2·86	13·2	14	26	204	508
	c	143	143	0	3·11	7·3	15	18	207	406
	d	121	132	+9	3·62	5·13	14	16	256	320
	*e**	130	108	−17	2·61	3·5	8	9	326	388
	*f**	110	96	−13	3·46	10·8	7	12	495	900
	*g**	106	90	−15	2·17	4·72	8	10	271	472

* After lung denervation

In the second type of experiment the lungs were ventilated artificially by an intermittent negative pressure applied to the thorax only. The results obtained from one experiment are shown in Fig. 5. When the carotid body chemoreceptors were stimulated by hypoxic blood, pulmonary ventilation remaining constant, the heart slowed from 126 to 62 beats/min (*A*). While the chemoreceptors were being stimulated, the rate and depth of artificial respiration were temporarily increased so as to mimic the effect occurring in the spontaneously breathing animal, and this resulted in an immediate increase in heart rate from 62 to 102 beats/min, which was maintained throughout the period of increased ventilation. This cardio-accelerator response to increased pulmonary ventilation was abolished by denervation of the lungs (Fig. 5*B*). It may also be noted that lung denervation enhanced the chronotropic effect of chemoreceptor stimulation.

The responses of the heart to single inflations of the lungs were also tested during perfusion of the carotid bodies with oxygenated blood or hypoxic blood. It was found that approximately doubling the inspiratory volume of the lungs caused a temporary tachycardia. This response occurred after dividing on each side the vertebral nerve, the rami communicantes T1–T4 and the sympathetic chain below T4, but was abolished by atropine or by denervation of the lungs only. This suggests that the cardio-accelerator responses are the result of a reflex from the lungs whose afferent and efferent pathways lie mainly in the vagus nerves.

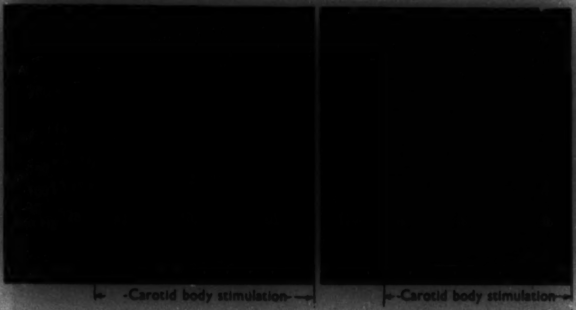

Fig. 5. Dog, ♂, 10·3 kg. Morphine–chloralose–urethane. Artificial respiration by negative pressure applied to the thorax only. Decamethonium iodide given to paralyse spontaneous respiratory movements before recording began. Left and right vagosympathetic nerves crushed below level of the lungs. Records show the effect of increased respiratory movements of the lungs on heart rate during stimulation of the carotid bodies by hypoxic blood before (*A*) and after (*B*) division of the left and right vagosympathetic nerves immediately above the hila of the lungs but below the origin of the cardiac branches of the vagus. Each section, *A* and *B*, shows the control heart rate before stimulation of the carotid bodies; then stimulation of the carotid bodies was begun and the trace commenced when a steady state had been reached; during the signals pulmonary ventilation was increased by increasing the negative pressure applied to the outside of the lungs and by raising the respiratory rate. The figures below the blood pressure record are those for heart rate (beats/min.)

The cardio-accelerator responses to single inflations of the lungs carried out during carotid body stimulation were not due to a lowering of the alveolar pCO_2 because a similar result was obtained if the single lung inflation was made simultaneously with changing the ventilation of the recipient animal from room air to a mixture of 5% CO_2 and 21% O_2 in N_2.

If the above interpretation of our results is the correct one, then it would be expected that a reduction or an abolition of the reflex hyperpnoea effected *during* a response to chemoreceptor stimulation would suppress the cardio-accelerator reflex originating in the lungs and so unmask the primary reflex bradycardia of chemoreceptor origin. Support for this contention was obtained in the following way. In spontaneously breathing dogs the carotid body chemoreceptors were stimulated by perfusion with hypoxic blood. This resulted in an increase in respiratory minute volume and, in different experiments, either in an increase or no change in heart rate. When a steady state had been reached, decamethonium iodide was given intravenously to reduce or abolish the hyperpnoea. This invariably caused a profound bradycardia. The typical effect is shown in Fig. 6. In this experiment stimulation of the chemoreceptors caused an increase in heart rate from 112 to 124 beats/min and in respiratory minute volume from 4·3 to 21·2 l./min. Injection of decamethonium during carotid body excitation resulted in an almost immediate reduction of heart rate from 124 to 30 beats/min and restoration of the respiratory minute volume to approximately its control value. The onset of the bradycardia occurred less than 10 sec after the injection and was coincident with the reduction in the depth of breathing as indicated by the record of intratracheal pressure. This chronotropic effect occurring in so short a time cannot be attributed to a change in cerebral blood pO_2 and pCO_2 consequent upon the change in pulmonary ventilation, because the brain was simultaneously perfused. The circulation time between the aortic arch and vertebral arteries in this experiment was about 1·5 min. Nor can the bradycardia be the result of asphyxial stimulation of the aortic arch chemoreceptors, because

Fig. 6. Dog, ♂, 16·2 kg. Morphine–chloralose–urethane. Closed chest; spontaneous respiration. Separate perfusion of cerebral circulation through the vertebral arteries. The cerebral perfusion pressure was maintained about 50 mm Hg higher than the systemic blood pressure but was not recorded. Between *a* and *c*, stimulation of the carotid bodies by hypoxic blood. During the period of stimulation, decamethonium iodide 5 mg was given intravenously at *b*. At arrow † positive pressure ventilation was applied. A period of 30 sec elapsed during the break in the records. The figures below the blood pressure trace are those for heart rate (beats/min).

the respiratory minute volume returned to its control value and, furthermore, it was not appreciably altered by applying artificial respiration (Fig. 6). Further control experiments carried out on anaesthetized dogs indicated that decamethonium itself has no appreciable effect on heart rate, provided pulmonary ventilation is maintained constant.

DISCUSSION

In much of the previously published work, reflexes from the carotid and aortic chemoreceptors were elicited by means of drugs such as acetylcholine, nicotine and lobeline injected into the blood stream. The possibility that the observed cardiovascular effects were complicated by concomitant stimulation of baroreceptors cannot be ruled out, because these drugs are known to excite not only chemoreceptors, but many other types of sensory nerve endings as well (Gray & Diamond, 1957; Green & Neil, 1958). In the present experiments, therefore, hypoxic blood from a donor animal was used to stimulate the carotid bodies. It should be mentioned, however, that in our preparations the carotid sinuses as well as the carotid bodies were perfused and that both were subjected to the same changes in chemical composition of the blood when the perfusate was changed. It is known that the sensitivity of the baroreceptors may be modified by various drugs applied to the wall of the carotid sinus through an alteration in distensibility of the arterial wall (Heymans & Neil, 1958, p. 72 *et seq.*). The question therefore arises as to whether the heart-rate responses observed in the present experiments were in any way dependent upon a similar mechanism resulting from changing the composition of the blood in the carotid sinuses. The evidence available suggests that this possibility is very unlikely. First, Bronk & Stella (1935) recorded the discharge of impulses from baroreceptors in single nerve fibres of the carotid sinus nerve and found that the relationship between the carotid sinus pressure and the impulse frequency was not altered by either a decrease in oxygen tension or an increase in carbon dioxide tension of the blood perfusing the sinuses. Diamond (1955) also found that the discharge from baroreceptors was not influenced by oxygen lack. Secondly, we have shown in other experiments (unpublished) that the changes in heart rate occurring in response to changing the perfusate from oxygenated to hypoxic blood were not appreciably modified by denervation of the barosensory area, the nerve supply to the carotid body being left intact. We believe, therefore, that the effects described in this paper as occurring in response to perfusing the carotid bifurcation regions with hypoxic blood are due solely to stimulation of the carotid body chemoreceptors.

Our results have shown that when the carotid bodies are stimulated in dogs with controlled pulmonary ventilation, slowing of the heart occurs and never acceleration. This response is independent of changes in carotid sinus pressure and systemic blood pressure and represents, therefore, the primary reflex effect on the heart. Similar results were obtained by Bernthal *et al.* (1951) and by Daly & Daly (1957) in experiments in which the carotid body chemoreceptors were stimulated by hypoxic or venous blood.

In the spontaneously breathing dog chemoreceptor stimulation not infrequently caused acceleration of the heart and the evidence presented indicates that this is not the result of a primary reflex from the carotid bodies, but is, at least in part, due to a secondary reflex arising from the lungs. It is also apparent from our experiments on spontaneously breathing dogs that this secondary reflex is operative in tests which resulted in slowing of the heart, for after lung denervation the bradycardia occurring in response to chemoreceptor stimulation was increased as compared with the control. It is evident, therefore, that stimulation of the carotid body chemoreceptors initiates two antagonistic reflexes, the balance of whose effects, among other factors, determines the directional change in heart rate.

The site of the antagonism between these two reflexes may be in the vagal centre itself. The primary reflex bradycardia occurring in response to stimulation of the chemoreceptors is abolished or reduced by division of the cervical vagosympathetic nerve and is probably due to stimulation of the vagal centre. The centre may be stimulated either directly by the chemoreceptors or indirectly by irradiation from the respiratory centre whose activity would be expected to increase although the animal may be ventilated artificially. This last possibility seems unlikely, because increased activity of the respiratory centre, produced by carbon dioxide for instance, causes, in the absence of reflex effects from the lungs, inhibition of the vagal centre, not stimulation (Anrep *et al.* 1936*b*). With regard to the secondary cardio-accelerator reflex arising from the lungs, the afferent and efferent pathways were shown to lie mainly in the vagus nerves so that this response must be brought about by inhibition of cardiac vagal tone.

We have not investigated the possible role of the sympathetic nervous system as an afferent or efferent pathway mediating the cardio-accelerator reflex. In this connexion, Bronk, Ferguson, Margaria & Solandt (1936) found that inflation of the lungs caused a decrease of impulse frequency in sympathetic fibres to the heart.

Reflexes arising from the lungs and affecting heart rate have been described previously. Knoll (1881) was the first to show that electrical stimulation of the pulmonary branches of the vagus nerve gave rise to cardio-acceleration and this finding was confirmed by later workers (von Saalfeld, 1932; Anrep *et al.* 1936*a*). It is, however, only with weak stimuli that this response can be elicited; strong stimulation of the same nerves caused bradycardia (Brodie & Russell, 1900; von Saalfeld, 1932; Anrep *et al.* 1936*a*). An increase in inspiratory volume of the lungs within a physiological range and produced either by a positive intrapulmonary pressure or by a negative intrathoracic pressure caused reflex cardio-acceleration (Hering, 1871; von Saalfeld, 1932, 1933; Anrep *et al.* 1936*a*) although larger inspiratory volumes caused reflex bradycardia (von Saalfeld, 1932, 1933; Anrep *et al.* 1936*a*). Both these reflexes have their afferent and efferent pathways in the vagus nerves. Another type of reflex arising from the lungs results from changes in pressure in the pulmonary vascular bed. Thus an increase in pulmonary arterial or pulmonary venous pressure caused slowing of the heart (Schwiegk, 1935; I. de B. Daly, Ludány, Todd & Verney, 1937; Aviado, Li, Kalow, Schmidt, Turnbull, Peskin, Hess & Weiss, 1951). We regard it as unlikely that the cardio-accelerator response observed in the present experiments is due to haemodynamic changes in the pulmonary circulation. Recent experiments have shown that cardio-acceleration occurs independently of changes in pulmonary arterial and venous pressures, and in intrathoracic blood volume measured by the Stewart dye-dilution method. It is more probable that it is due to a stretch reflex from the lungs, brought about by the increase in tidal air volume. The most convincing evidence for this was the finding that, in experiments in which the lungs were artificially ventilated by a negative pressure applied to the thorax, chemoreceptor stimulation caused an increase in cardiac vagal tone which was temporarily reduced by increasing the inspiratory volume of the lungs for a single breath only or by a hyperpnoea to mimic the ventilatory response occurring during carotid body stimulation. This reduction of vagal tone did not occur after denervation of the lungs. These results obtained during carotid body stimulation are therefore similar to those reported by Anrep *et al.* (1936*a*, *b*) using the innervated heart-lung preparation of the dog. They presented evidence that the lungs are a constant source of afferent impulses inhibiting the vagal centre. These cardio-accelerator impulses are minimal, but not absent, during deflation of the lungs and are greatly increased during lung inflation and bring about inhibition of the vagal centre. There is, however, another way in which an increase in the number of inhibitory impulses reaching the vagal centre from the lungs may be evoked, that is by an increase in the frequency of respiration *per se.* Since stimulation of the carotid bodies causes an increase in rate as well as in depth of respiration, this may be a contributory mechanism by which cardio-acceleration is effected. We have not, however, made any experiments to test this hypothesis. Several types of respiratory receptor which are excited by inflation of the lungs have been described (Adrian, 1933; Widdicombe, 1954) but there is, as yet, little information as to the identity or location of the receptors giving rise to cardio-accelerator effects.

In the present experiments the recipient animal breathed room air and therefore the medullary centres received fully oxygenated blood. Neil (1956) has recently emphasized the importance of eliciting chemoreceptor reflexes with a background of systemic hypoxia when their role in the production of the tachycardia by hypoxia is being studied. With this view we are in general agreement. The purpose of the present investigation was to study, under controlled conditions, some of the mechanisms responsible for the variable effects on heart rate resulting from stimulation of the carotid bodies. These conditions can be more easily controlled with the animal breathing room air than a gas mixture of low oxygen content.

It is evident from our own results and from those of Bernthal *et al.* (1951) and of Neil (1956) that the tachycardia which occurs in systemic hypoxia cannot be attributed to a primary reflex from the carotid bodies. The possibility that a pulmonary reflex of the type described here may contribute to this response requires further study.

SUMMARY

1. An investigation has been made of the effects of stimulation of the carotid body chemoreceptors on heart rate in anaesthetized dogs breathing room air. The chemoreceptors were stimulated by changing the perfusate from oxygenated to hypoxic blood.

2. In preparations with open or closed chest, artificially ventilated at a constant rate and tidal volume, stimulation of the carotid bodies invariably caused a profound bradycardia, which occurred independently of changes in systemic blood pressure and carotid sinus pressure. The bradycardia was reduced or abolished by atropine or by division of the cervical vagosympathetic nerves and was abolished by division of the carotid sinus nerves.

3. In dogs spontaneously breathing room air, chemoreceptor stimulation caused either an increase, a decrease or no change in heart rate. The respiratory minute volume invariably increased.

4. Evidence is presented that the tachycardia occurring in spontaneously breathing dogs in response to carotid body stimulation is due, at least in part, to a reflex originating in the lungs. The afferent and efferent pathways for this reflex lie mainly in the vagus nerves. The possible nature of the reflex is discussed.

5. Our results indicate that chemoreceptor stimulation by hypoxic blood causes a primary reflex bradycardia and that this effect is partly or wholly masked by a secondary reflex tachycardia originating in the lungs.

We wish to express our thanks to Mr D. A. Sholl for statistical advice, to Mr D. R. Bacon for technical assistance, and to the Royal Society for a grant to one of us (M. de B. D.) defraying part of the expenses of this work.

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THE BEHAVIOUR OF FROG MUSCLE IN HYPERTONIC SOLUTIONS

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It is well known that hypertonic solutions diminish, and in sufficient concentration abolish, the twitch of frog muscle, whereas *hypotonic* solutions within a certain range enhance the twitch (Overton, 1902; Fenn, 1936; Hodgkin & Horowicz, 1957). This paper describes some mechanical measurements made with muscles exposed to solutions up to the equivalent of three times the concentration of normal Ringer's solution, and to *hypotonic* solutions.

METHODS

All the experiments described were made with sartorii of English frogs, male and female: no seasonal variations were noticed. Similar results were obtained with the sartorii of toads.

Most of the experiments involved measurement of the speed of shortening or of the tension developed. Shortening was recorded by means of a light isotonic lever of low inertia bearing a vane which cast a shadow on a twin phototube (Hill, 1951). The potential change arising from movement of the shadow was amplified by a Kelvin and Hughes amplifier Type 6 and displayed on a smoked drum by a Kelvin and Hughes pen recorder Mk. V. Tension was recorded by means of an R.C.A. 5734 transducer triode and similarly displayed on a smoked drum or, when high amplification and speed were required, photographically from a cathode-ray tube.

In early experiments muscles were mounted on a multi-electrode assembly to eliminate the effect of any possible change in the rate of propagation of the contractile wave. This was later found unnecessary and a simple holder bearing two platinum electrodes was adopted, exposing both faces of the muscle to the solution, thus allowing quicker equilibration. Stimulation was direct.

The normal Ringer's solution was: (mM) NaCl, 115·5; KCl, 2·5; $CaCl_2$, 1·8. *Hypotonic* solutions were made by adding the calculated amount of distilled water. Hypertonic solutions were of two kinds in which the excess osmotic pressure was provided, (*a*) by sucrose and (*b*) by salts. 'Sucrose hypertonic Ringer' was made by dissolving solid sucrose in normal Ringer's solution, assuming 220 mM-sucrose to be isotonic. Salt-enriched solutions were made by dilution of stock solution of 10 × the concentration of standard Ringer's solution in all constituents. Buffering was provided by 2 mM sodium phosphate buffer at pH 7·2. Throughout this paper concentrations are expressed relative to Ringer's solution, e.g. 2·5 × R, or 0·7 × R. Where the word '*hypotonic*' appears it is italicized to make it more easily distinguished from 'hypertonic'.

Experiments were made at 0° C and in the region 18–20° C. There was no qualitative difference between results at the two temperatures.

RESULTS

The concentration of solutions used was often very far from that of Ringer's solution (from one half to three times) and the muscles underwent considerable shrinking or swelling. They suffered little or no permanent damage by such treatment, and recovery was rapid in normal Ringer's solution, even after 6 hr exposure.

Velocity of shortening

Isometric twitches recorded while a muscle is equilibrating with a hypertonic solution show a progressive decline of peak tension, and a later and slower take-off. This suggested that the velocity of shortening was reduced. The first experiments were made to examine that possibility.

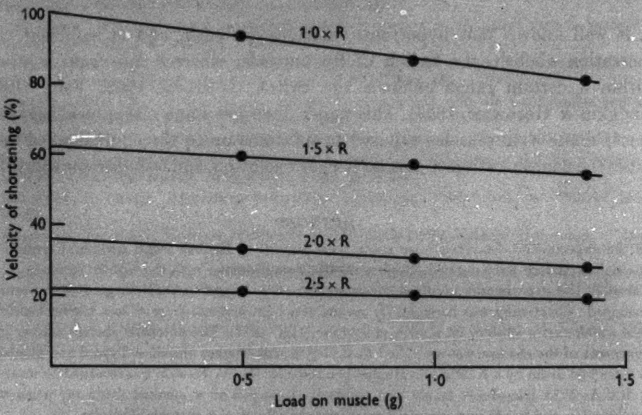

Fig. 1. Velocity of shortening as a function of load in tetanic contraction at 19° C. Muscles exposed to concentrations shown on curves. Velocity expressed as percentage of maximum velocity in Ringer's solution.

Speed of shortening depends on the load, and on the strength of a muscle, except for zero load when speed is independent of strength: so to avoid errors due to feebler contraction in hypertonic solutions, it was necessary to measure speed at zero load. The condition of zero load cannot be realized experimentally, so velocity has here been measured with the muscle shortening against very small loads (0·5–2 g). From these values a portion of the load–velocity curve was extrapolated to give velocity at zero load (intrinsic speed). The intrinsic speed so obtained was greatly diminished by hypertonic solutions. For the results shown in Fig. 1 different muscles were used for the different concentrations, so the velocity is expressed as percentage of the velocity at zero load in normal Ringer's solution. In every case the muscles regained their original speeds within 15 min of being returned to normal Ringer's solution; recovery is, in fact, nearly complete in 5 min. Fig. 1 was obtained from experiments in which the concentration was raised by electrolytes, but similar results were obtained in sucrose hypertonic Ringer.

Experiments made with *hypotonic* solutions showed no effect on the velocity of shortening.

Isometric tension

The strength of a muscle is diminished in hypertonic solutions. At body length the isometric tension in a tetanus was reduced, in 2½ × R, to about 30 % of that in Ringer's solution. Fig. 2 shows the relation between tetanic isometric tension of a single sartorius and the concentration of the bathing fluid. As was expected from observations of the speed of shortening, tension developed extremely slowly in concentrated solution. In 2½ × R a 6 sec tetanus was needed to achieve maximum tension at room temperature and a much longer one at 0° C.

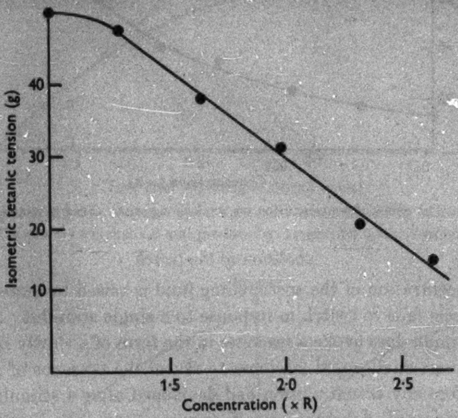

Fig. 2. Effect of concentration on the maximum isometric tension of frog sartorius. Tetanus; 18° C.

Isometric tetanic tension, like velocity of shortening, is not changed by *hypotonic* solutions down to about 0·6 × R.

Latent period

If part of the latent period represents the time needed for a muscle to take up internal slack, it was to be expected that, in hypertonic solutions, when the velocity of shortening is reduced, the latent period would be extended: this proved invariably to be the case. The latent period was measured by recording tension in the early part of a twitch with high amplification and measuring the time from stimulus to the first detectable rise of tension. Fig. 3 is a curve of latent period against concentration. No attempt was made to measure latency relaxation, but Professor A. Sandow has informed Professor A. V. Hill that latency relaxation could not be detected in a muscle after the twitch had been abolished by 3 × R.

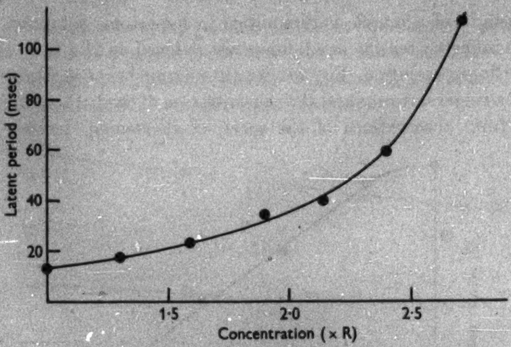

Fig. 3. The effect of concentration on the latent period. Frog sartorius; 0° C.

Abolition of the twitch

If the concentration of the surrounding fluid is raised to more than 2½ × to 3 × R, a muscle fails to twitch in response to a single stimulus. Nevertheless, a series of stimuli does evoke a response in the form of a slowly rising tension. Hill (1950) used small, rapid stretches to reveal the presence of a mechanical response before any actual tension had developed after a stimulus. The same technique was applied here to see if any mechanical response to a single stimulus could be detected after the usual twitch response had been abolished by hypertonic solution. Tension was recorded by means of a transducer mounted on the arm of a Levin–Wyman ergometer (Levin & Wyman, 1927) which applied small stretches to the muscle soon after the stimulus. A mechanical response showed as an increased resistance to stretch. The amount and speed of the stretch were varied and in each case the tension in a stimulated muscle was compared with that for a similar stretch without a stimulus. Each stretch began from the same initial length, the muscle having been previously tetanized at that length to take up any slack remaining from a previous stretch. Sometimes the stretch with stimulus preceded that without, sometimes the reverse. The result was always the same; the muscle exhibited greater resistance to stretch when stimulated than when not stimulated. Fig. 4 is a typical result of such an experiment. This clearly demonstrates the presence of a mechanical response but provides no measure of it. It was possible that the slow rise of tension in a tetanus after abolition of the twitch was due to some sort of recruitment of activity by the successive stimuli. To test this the tension was recorded during a tetanus in which the muscle was suddenly released a few millimetres when the tension was high; the redevelopment of tension was then compared with the initial development. Fig. 5 is a typical record where it is seen that redevelopment, though not so slow as the original rise of tension, was still very slow compared with that of muscles in normal Ringer's solution.

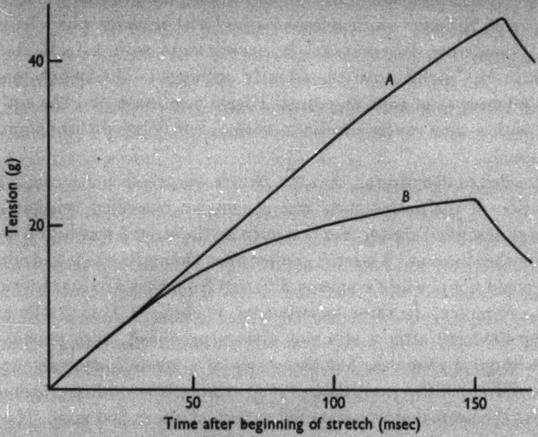

Fig. 4. Tension during 2·3 mm constant speed stretches; *A* stimulated by single shock, *B* not stimulated. The muscle was in 3 × R and produced no tension for a single stimulus without a stretch.

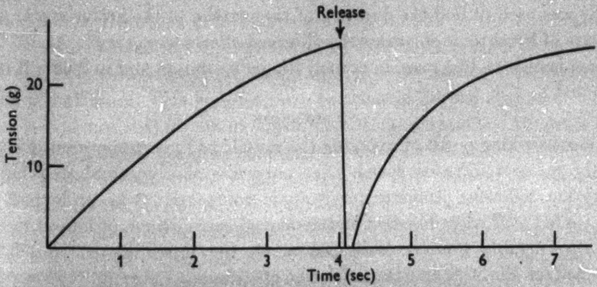

Fig. 5. Tension during a maintained tetanus showing redevelopment after a quick release of 2·3 mm. Frog sartorius in 3 × R.

Duration of the active state

Conventional methods for measuring duration of the active state (Hill, 1953; Macpherson & Wilkie, 1954; Ritchie, 1954) are not applicable to muscles in the more concentrated solutions because of their extreme slowness, but it is possible to use Ritchie's quick-release method with muscles which have undergone only moderate dehydration. In experiments with 1·5 × R at 0° C the active state was slightly and consistently prolonged. The twitch reached its peak 40–60 msec later than in normal Ringer's solution and the active state decayed with a time course similar to normal but delayed throughout by the same amount.

For muscles so dehydrated that the twitch was abolished a rough guide to the duration of the active state was sought by recording tension in quick stretches as described above. For this purpose the stretch was begun at various times after the stimulus. A stretch applied after the active state had completely decayed could not produce a tension different from that produced by a stretch without a stimulus. In these experiments, made at 0° C in 3 × R, a stretch beginning 400 msec after a stimulus always produced more tension than a stretch without a stimulus, but the result of a stretch after an interval of 600 msec did not differ from that of one without a stimulus. Ritchie (1954) found that the normal active state fell to zero at about 500 msec. The present experiments, then, do not show any marked effect of hypertonic solutions on the duration of the active state.

For muscles in *hypotonic* solutions the method of Macpherson & Wilkie (1954) was used to find the duration of the plateau of the active state. As in the case of hypertonic solutions the observed effects were small. At 18° C the plateau lasted for 16·5 msec in normal Ringer's solution and in 0·62 × R it was extended to 21·8 msec.

DISCUSSION

The most striking result of exposing the muscle to hypertonic solutions is the greatly reduced intrinsic speed. Not only was shortening slowed but the relaxation following isometric or isotonic contraction was prolonged. The muscles felt stiff when handled and displayed some degree of lateral rigidity. The considerable tension developed when the unstimulated muscle was stretched (cf. Fig. 4) is another indication of stiffness; a normal frog's sartorius would have developed little or no tension for a similar stretch. All these facts give the impression of a greatly increased viscosity.

One way in which such enhanced viscosity could arise is by close packing of the I and A filaments (Huxley, 1957) and other formed elements of the fibre. As water is withdrawn and the fibre diameter shrinks some packing must occur which may lead to friction between parallel sliding structures. Dr H. E. Huxley kindly prepared some sections for the electron microscope, but these did not furnish any new information because of the expected difficulty, that shrinkage during fixing and staining was greater than that in the most concentrated solutions used during the present experiments.

Another factor to be considered is the new ionic concentration within the fibre which must initially increase in proportion to water loss. It would not be surprising if the contractile proteins were seriously affected by this change. Solutions of actomyosin and actomyosin threads are very sensitive to their ionic environment (Perry, 1956). For example, aqueous solutions of G-actin which are quite mobile polymerize and become viscous with the addition of univalent ions. Further, the ability of actomyosin threads to complete their cycle of contraction and relaxation depends on the relative concentration of calcium and magnesium ions. The decline of isometric tension may also be a result of the changed ionic concentrations within the fibre.

There is no doubt that at the higher concentrations the normal isometric twitch is entirely abolished (Fenn, 1936), although the action potential persists (Hodgkin & Horowicz, 1957; Ishiko & Sato, 1957). At first sight this suggested that the link between action potential and contractile process had been broken. The stretch experiments described above do not support that view. They reveal a mechanical response to a single stimulus which implies that the link is intact. The latter view is supported by the observation (Hill, 1958) that the 'activation heat', that part of the heat in a twitch which accompanies the setting up of the active process, is substantially unchanged by hypertonic solutions even when the twitch is absent and there is no shortening heat or work.

Hodgkin & Horowicz (1957) have drawn attention to the slowness of the rise of tetanic tension in dehydrated muscle. This would result if the active state was not fully established by a single stimulus but needed several stimuli to reach a maximum. This is not known to occur in normal frog sartorius, but there is evidence that it does in lamellibranch smooth muscle (Abbott & Lowy, 1958; B. R. Jewell, personal communication). The release experiments were made to see if such recruitment caused the slow tension development in hypertonic solutions. The release occurred when the tension, and therefore the activity, was maximal so subsequent redevelopment of tension would not then have to wait upon the further accumulation of activity but would rise rapidly to its former level. In fact this did not happen; redevelopment was very slow (Fig. 5). This indicates that recruitment of activity is not responsible for the slowness of the rise of tension in a tetanus. That is not to say that there was no recruitment at all; in fact the redevelopment was always, as in Fig. 5, slightly quicker than initial development of tension. However, that is also seen in normal frog muscle (Jewell & Wilkie, 1958), and Hill's observation of the full activation heat (Hill, 1958) for a single stimulus in 3 × R tells against any idea of recruitment.

The duration of the active state, so far as it can be measured in hypertonic Ringer, is not much different from that of normal muscle. The present experiments also fail to show appreciable change in *hypotonic* solution. In some other experiments, when duration of the active state was not being measured, I have seen considerable increase of twitch tension and duration when the Ringer's solution was diluted. This was not due only to the paucity of ions, for the effect was reversed by making up the concentration again with sucrose. Other authors have recorded similar observations (Fenn, 1936; Lullmann & Muscholl, 1954) and it is tempting to explain them by supposing a prolonged active state. However, under the conditions of the present experiments the prolongation is too small to allow that explanation.

The frog's sartorius in hypertonic solution resembles certain smooth muscles in some of its properties, e.g. the slowness of contraction and relaxation and the response to stretch. Unlike the normal sartorius, the muscle in hypertonic solution shows considerable resistance to small stretches without a stimulus (Fig. 4). This is typical of many smooth muscles, notably those of the marine lamellibranchs. Also, as in these muscles, the decay of tension following such stretch, i.e. stress relaxation, is very long-lasting. Qualitatively at least the muscle in hypertonic solution obeys the relation described by Abbott & Lowy (1957) for several smooth muscles, i.e. the rate of stress relaxation increases with intrinsic speed.

SUMMARY

1. The mechanical properties of frog's striated muscle have been examined in solutions of different concentrations.

2. The velocity of shortening is greatly reduced by hypertonic solutions but is not affected by *hypotonic* solutions down to half the concentration of normal Ringer's solution.

3. The maximum tetanic tension is reduced by hypertonic solutions but not affected by *hypotonic* solutions.

4. In solutions of 2½–3 times the concentration of normal Ringer's solution the twitch response to a single stimulus is abolished. Nevertheless, the action potential still provokes activity in the contractile proteins. It is suggested that failure to develop tension to a single shock is due to the great slowness. The muscle is not able to extend the series compliance sufficiently in the brief period of the active state.

This work was undertaken during tenure of a grant for scientific assistance made to Professor A. V. Hill by the Medical Research Council. I welcome this opportunity to express my gratitude to Professor Hill for his encouragement and advice.

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MEMBRANE PERMEABILITY CHANGE DURING INHIBITORY TRANSMITTER ACTION IN CRUSTACEAN MUSCLE

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Impulses in the inhibitory nerve fibre to the limb muscle of the crustacean have been shown to increase the conductance of the post-junctional muscle fibre membrane without there being, in the absence of an applied current, an appreciable change in membrane potential from the resting level (Fatt & Katz, 1953*b*). This increase in membrane conductance may be taken to indicate an increase in membrane permeability toward some ions in the neighbourhood, and the fact that the equilibrium potential at which there is no net transfer of charge is close to the resting potential suggests that the ions involved are potassium or chloride, both of which will probably be in a condition of equilibrium across the resting membrane. The experiments reported in this paper were designed to establish toward which of these two species of ions the permeability is increased. The procedure was to examine the effect on the inhibitory response of variation in the concentrations of K^+ and Cl^- ions in the solution bathing the muscle. A subsidiary problem was to determine the action of γ-aminobutyric acid on the membrane properties.

METHODS

Preparation. Electrical records were obtained from fibres of the opener muscle of the claw of the crayfish, *Astacus fluviatilis*, in response to stimulation of its inhibitory nerve fibre. The claw was removed from the animal at the point of natural autotomy. The muscle was exposed by opening the shell of the propodite and removing the closer muscle, this operation being carried out with frequent washing to avoid the deleterious action of substances issuing from damaged muscle. The nerve was exposed in the meropodite and separated into its constituent bundles (usually four) which were tested for the presence of the one inhibitory and one excitatory nerve fibre which are known to supply the opener muscle (Hoffman, 1914; van Harreveld & Wiersma, 1937). The cut central ends of the nerve bundles were stimulated separately by drawing them into narrow glass tubes and applying brief pulses of current between the interior of these tubes and the surrounding fluid.

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The prepared claw was fixed in the experimental chamber and immersed in about 7 ml. of fluid. The bathing fluid was changed by emptying the chamber through an outlet at the bottom and refilling it with fresh fluid, this procedure being repeated three or more times before starting measurements in a solution of altered composition.

Electrodes. Two intracellular micro-electrodes were employed. A constant current was applied through one, while the steady level of potential and the potential changes produced by nerve stimulation were recorded by the other. In preliminary experiments the micro-electrodes were filled with 3M-KCl. The impression was received that with such electrodes the response to stimulation of the inhibitory fibre altered with time, as might be expected if Cl were diffusing out of the electrodes and if the inhibitory impulses caused an increase in membrane permeability toward this ion species (cf. observations on the motoneurone, Coombs, Eccles & Fatt, 1955). Although the effect was not conclusively demonstrated in a controlled experiment with electrodes filled with different salts, it was decided to avoid this source of uncertainty by filling the electrodes with salts of large anions. Accordingly, most experiments were done with electrodes filled with either 2M-K acetylglycine or 2M-Na or K citrate. Electrodes filled with a citrate salt (in some cases neutralized with citric acid) were particularly useful for applying current. When a KCl- or K acetylglycine-filled electrode was used, it was commonly found that after its insertion into a muscle fibre the maximum outward current that could be passed was reduced to a very small value compared with that which could be passed with the electrode outside. With citrate-filled electrodes this difficulty was appreciably reduced, without the electrical properties of the fibre being affected (Fatt & Ginsborg, unpublished observations). A possible explanation is that citrate ions released inside the muscle fibre caused the decomposition of insoluble Ca compounds which would otherwise migrate into the electrode tip and block it when it was made positive.

Two large electrodes were used to make contact with the bathing fluid; one was connected to the input of the balanced voltage-recording amplifier, and the other was included in the circuit for applying current (see Fatt & Katz, 1953*a*). These electrodes were constructed with a bridge of 0·24M-NaCl-agar gel separating the bathing solution from a Ag–AgCl junction. Changes in liquid junction potential would have occurred at the recording bath electrode whenever the ionic composition of the bathing solution was varied, and the recorded membrane potentials had to be corrected for these changes. Differences in liquid junction potential, occurring on changing between different solutions, were determined by observing the potential between the normal bath electrode and a coarse 3M-KCl-filled capillary electrode (resistance under 1 MΩ). As a result of these measurements a correction of −8·5 mV was applied to membrane potentials recorded in a K- and Cl-free solution (Cl replaced by pyroglutamate or acetylglycine, Figs. 3, 4, 6 and 7) and −9·0 mV for membrane potentials recorded in a 20 mM-K, Cl-free solution (Fig. 6).

The absolute values of membrane potential have been calculated on the basis of the potential recorded when the electrode was withdrawn from the fibre at the end of each experiment, with the bathing solution having the normal Cl content. Since the recording micro-electrode was ordinarily not filled with KCl, the difference in junction potential at its tip when in the bathing solution from when inside the fibre may have been considerable. No attempt has been made to correct for this. Further inaccuracy arose from the fact that in some experiments the electrode was not withdrawn until over an hour after the significant measurements of the inhibitory response had been made. The absolute values given for the membrane potentials are therefore less accurate than changes of potential, and may be up to 15 mV in error. Drift in the recording system appeared to be mainly in the electrodes, and may be taken to have been less than 5 mV in the experiments reported here, none of which lasted more than 1 hr.

Solutions. The standard bathing solution was that of van Harreveld (1936) containing (mM) Na 210, K 5·25, Ca 14·0, Mg 2·8, Cl 247 and HCO_3 2·1. In some experiments the Mg was omitted, and in some the Ca concentration was raised to 28 or 32 mM. The ions whose effects on inhibitory actions were investigated were K and Cl. When the K concentration was varied (between 0 and 20 mM), compensatory changes were made in the concentration of Na to maintain a constant osmotic pressure. Variation in the Cl concentration was effected through its replacement by equivalent amounts of pyroglutamate or acetylglycine. These organic anions were used because it was assumed that their large size would prevent their penetrating the surface membrane of the muscle fibre. Pyroglutamate has been found to be a useful substitute for Cl; unlike glutamate, it does not cause a large increase of membrane conductance, possibly through the removal of ionized Ca (Fatt & Ginsborg, unpublished observations). Acetylglycine was less satisfactory; when used to replace Cl in van Harreveld solution, it caused repetitive nerve discharges to occur in response to single stimuli, as well as spontaneous discharges. Experiments could still be conducted with acetylglycine, provided the Ca concentration was raised to about twice that in van Harreveld solution.

Experimental procedure. After the prepared crayfish leg had been placed in the experimental chamber, the two micro-electrodes were inserted into a muscle fibre not more than 0·2 mm apart, and, unless they were accidentally displaced, remained inserted throughout an experiment. The previously prepared nerve bundles were tested by repetitive stimuli to locate the inhibitory and, if it was also possible, the excitatory nerve fibre supplying the muscle. The inhibitory nerve fibre was identified either through its ability to effect a large reduction in the amplitude of the excitatory junctional potential, or through the appearance of an inhibitory junctional potential when the membrane potential was displaced from its resting level (Fatt & Katz, 1953*b*). When examining the effects of junctional action, the nerve was stimulated repetitively at a frequency of 20–25/sec.

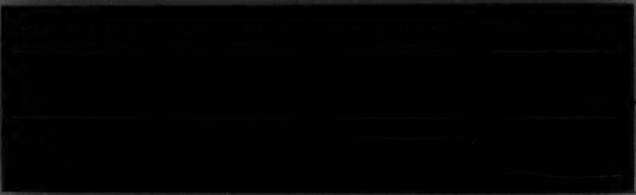

Fig. 1. Response of muscle fibre to inhibitory nerve fibre stimulation at various levels of membrane potential. Preparation bathed in normal van Harreveld solution. Membrane potential and applied current indicated on each record. Upward displacement represents depolarization. Stimulus artifact produces a gap in the sweep. See Methods, Experimental procedure.

One beam of a double-beam oscilloscope was used for recording current, and the other voltage; the latter allowed potential differences to be read to 0·05 mV. In addition, a constant voltage source with decade stages of 10 and 1 mV was used to offset approximately the membrane potential. Before commencing to record photographically, the current was turned on, displacing the membrane potential to a new level, and the nerve was stimulated for a few seconds. This allowed time for the pre-junctional facilitatory process to reach a steady condition, as well as for summation of successive junctional potentials to a steady level. After recording superimposable sweeps (except for random fluctuation) for about 2 sec, the stimulus was terminated and the photographic record continued until the potential had returned to a steady level and remained there for about 2 sec. Examples of records obtained by this procedure are shown in Fig. 1. All records were qualitatively similar to these.

Three factors limited the amount of information which could be obtained experimentally. (1) There was the purely technical difficulty of passing constant current through the micro-electrode, which was most troublesome for outward current. (2) Contraction occurred when the muscle fibre was depolarized beyond a certain level, resulting in injury around the point of impalement and loss of resting potential; the junctional potential–membrane potential relation, however, was often found to be only slightly affected. (3) Most experiments were terminated by a sudden, uncontrollable failure of conduction in the nerve fibre. The block occurred neither close to the point of stimulation, since it was unaffected by stimulus strength, nor close to the pre-junctional terminals, since the response failed in an all-or-none manner in the muscle fibre, and possibly in the whole muscle.

Presentation of data. The amplitude of junctional potential plotted in Figs. 2–7 represents the difference between the final steady level of potential and the maximum of the standing wave produced during nerve stimulation. The potential difference thus measured will depend in a complicated way on the conductance characteristics of the non-junctional membrane. If one could neglect the reactance of the membrane, and if the muscle fibre were acted on uniformly by the applied current and by junctional action, it would be possible to analyse the effect of transmitter action on the basis of the conductance of the junctional and non-junctional regions of membrane in parallel. As it is, these conditions do not apply (except possibly the uniform distribution of junctional action), and only a qualitative analysis is practicable. Summation of the potential changes produced by successive nerve impulses was an important factor in increasing the size of the measured junctional potentials, and thus improving the accuracy with which the reversal potential could be determined. This meant that the measured amplitude of the inhibitory potential was highly dependent on the time constant of the membrane and hence on the membrane conductance. This is noticeable in the plotted results.

A complication in the interpretation of the level of membrane potential at which the inhibitory potential reverses arises from the fact that the displacement of membrane potential by current is not uniform along the fibre, but decreases with increasing distance from the position at which current is applied (and potential recorded). The complete analysis to evaluate the equilibrium potential as described by Burke & Ginsborg (1956) was not feasible here, because the non-linear conductance of the fibre membrane makes an accurate estimation of the effect of the non-junctional membrane impossible. However, since in the present study the changes of reversal potential usually occurred around the resting potential, conclusions relating to the role of different ion species can be drawn from the direction of the reversal potential from the resting potential, which will be the same as for the equilibrium potential.

RESULTS

Behaviour in normal and K-free solution

In a muscle that was dissected and subsequently examined in a solution with normal K and Cl concentrations there was usually very little potential change evoked by stimulation of the inhibitory nerve fibre. On displacing the membrane potential by a steady current, an inhibitory junctional potential was recorded, which, depending on the displacement, could be in either direction, there being a reversal potential close to the resting potential toward which the inhibitory response was directed (Fig. 1; Fatt & Katz, 1953*b*). A plot of the amplitude of the inhibitory potential against the level of membrane potential from which it arose gave an S-shaped curve. Not only did the slope of this curve decline at high and low membrane potentials, but it frequently reversed. The non-linearity of the inhibitory potential–membrane potential curve can be accounted for to a large extent by variation in the resistance of the non-junctional membrane in parallel with the region generating the junctional response.

The voltage–current curve from which the conductance characteristics of the non-junctional membrane is inferred had the shape described by Fatt & Ginsborg (1958). The slope in the neighbourhood of the resting potential (zero current) was of the order of 100 kΩ. The slope decreased when the potential was displaced more than about 10 mV in either direction. In addition, following the application of an inward current, hyperpolarizing the membrane, the potential returned to a resting level which was a few millivolts higher than initially, and following an outward current the potential returned to a lower level. (The latter change was distinguishable from the more drastic loss of resting potential that occurred when depolarization caused contraction with consequent injury.) The extent of the alteration in resting potential depended on the duration of the current, though there did not appear to be any subsequent recovery. This effect was the main cause of the scatter in the plotting of the voltage–current relation. It may have been caused by a change in the relative permeability of the membrane to different ions, or, alternatively, by a change in the concentration of ions at the inner boundary of the membrane.

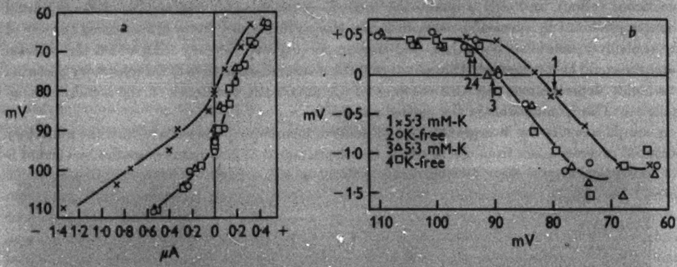

Fig. 2. *a*, Plot of voltage–current relation; *b*, plot of inhibitory junctional potential–membrane potential relation. Arrows indicate the average resting potential for each set of measurements as numbered. Solutions were van Harreveld solution and its K-free modification, observations being made in these solutions in the order indicated. Outward current is positive. Depolarization produced during inhibitory action is positive.

Changing from normal van Harreveld solution (5·25 mM-K) to a K-free solution caused the resting potential to rise by 10–15 mV. The reversal potential also rose (in three out of four experiments), but by a smaller amount than the resting potential, so that, whereas in normal van Harreveld solution the reversal potential was close to the resting potential, in the K-free solution it lay appreciably below the resting potential. As is shown in Fig. 2, the effect of K removal was not reversible: on returning to normal van Harreveld solution neither the resting potential nor the reversal potential returned to their original levels. The irreversibility of the effect of K-removal on the resting potential was already reported by Fatt & Katz (1953*a*). It probably has an origin similar to that of the incomplete return of potential following the application of current as described above. It appears probable from the results described below that the irreversible change in reversal potential is due to an alteration in the internal Cl concentration, which in turn is determined by the membrane potential.

Effect of Cl removal

Replacement of Cl by organic anions caused a loss in resting potential of usually 10–20 mV. Except for a displacement in the voltage scale the voltage–current curve was unaltered. Despite the fall of resting potential, the response to inhibitory nerve stimulation now appeared as a transient depolarization. Its time course was the same as under other conditions. On the displacement of the membrane potential it was found that the inhibitory potential did not reverse to a hyperpolarization until the membrane potential was reduced to beyond 10 mV below the resting level. Although there were technical difficulties in producing the necessary displacement, it was usually possible to reverse the response. In Fig. 3 the washing with Cl-free solution may have been incomplete and the reversed inhibitory potential elicited at low membrane potentials may have been due to some Cl remaining in the bathing solution. In the experiment of Fig. 4, 12 min after the first set of measurements in Cl-free solution the bathing solution was changed again (three washings with Cl-free solution) and further measurements were made. As can be seen, the additional washing caused only a small further change. This does not, however, eliminate the possibility that the reversed response at low membrane potential, which was here very small, may have been due to an inward movement of Cl. There will evidently be a continual outward leakage of Cl from the muscle fibres in Cl-free solution, and the accumulation of some of this Cl in the space surrounding the fibre may have been sufficient to give the present result. This is supported by the observation that when the large outward current required to give the inhibitory potential of reversed polarity was maintained for more than about 15 sec the response reverted to a transient depolarization without the level of the membrane potential altering, which may be explained by the reduction in the rate of loss of Cl from the fibre at the lowered membrane potential.

The reversibility of the effect of Cl-free solution is illustrated in Fig. 3. The reduction in the inhibitory potential occurring at high membrane potentials, despite an increased membrane resistance (indicated by the increased slope of the voltage–current curve), may be accounted for by a depletion of internal Cl during soaking in Cl-free solution (p. 186).

Effect of high K concentration

A large increase in K concentration (from 0 to 20 mM) produced an approximately reversible fall in resting potential. In the experiment of Fig. 5 responses to stimulation of both the excitatory and inhibitory nerve fibres were recorded. The addition of 20 mM-K caused a fall in resting potential of 30 mV. The slopes of the voltage–current curve around the resting potential simultaneously fell to one-third. Observed in the absence of applied current (i.e. at the resting potential level) the inhibitory response changed from a very small depolarization in K-free solution to a large hyperpolarization in 20 mM-K. On examining the response over a range of membrane potentials the reversal potential was found to be nearly unchanged. It should be noted, however, that the equilibrium potential of inhibition (which will be closer to the resting potential) will have shifted to a somewhat lower membrane potential in 20 mM-K.

The excitatory junctional potential in K-free solution had a maximum about 7 mV below the resting potential, and it fell rapidly with increasing membrane potential, corresponding to a decline in membrane resistance as seen in the voltage–current curve. In 20 mM-K the excitatory potential had a nearly

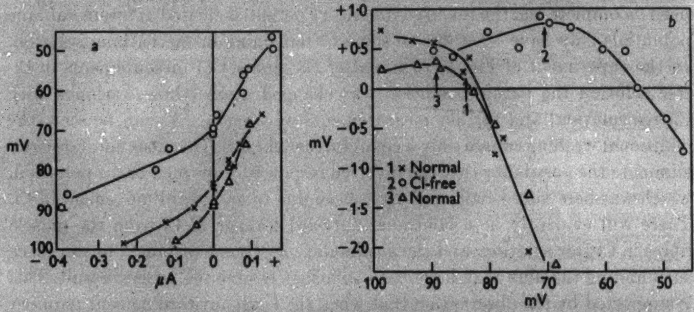

Fig. 3. *a*, Voltage–current relation; *b*, inhibitory junctional potential–membrane potential relation. Solutions consisted of 192 mM-Na, 32 mM-Ca with the anions (besides about 2 mM-HCO_3) either Cl (normal) or acetylglycine (Cl-free).

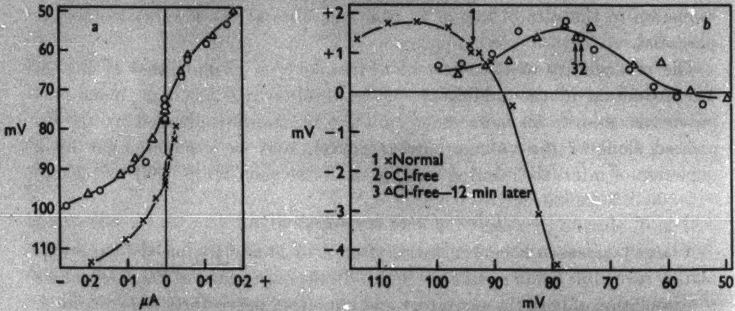

Fig. 4. *a*, Voltage–current relation; *b*, inhibitory junctional potential–membrane potential relation. Solutions consisted of 201 mM-Na, 28 mM-Ca, 2·8 mM-Mg with the anions either Cl (normal) or acetylglycine (Cl-free). Second set of measurements was obtained 1–2 min after changing to Cl-free solution; third set 12 min later, following further washing.

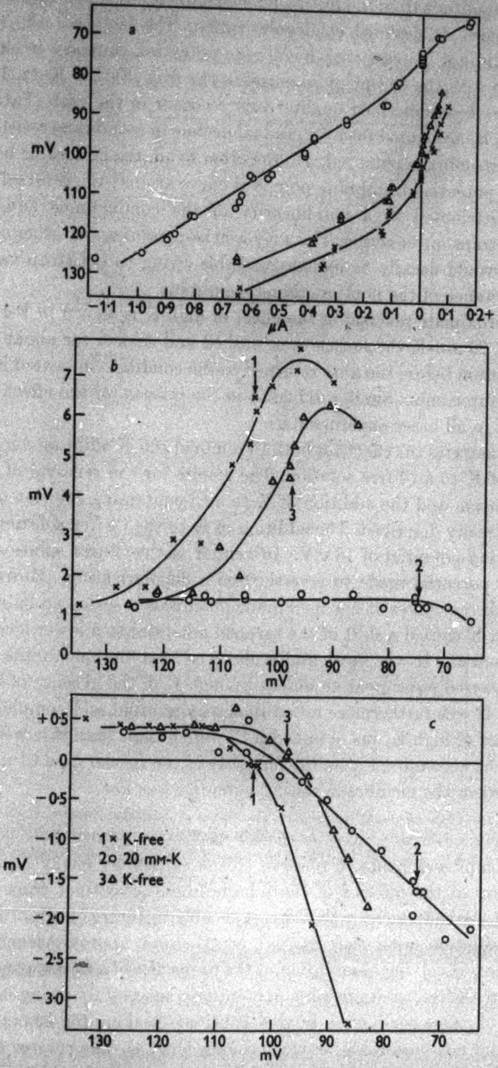

Fig. 5. *a*, Voltage–current relation; *b*, excitatory junctional potential–membrane potential relation; *c*, inhibitory junctional potential–membrane potential relation. Solutions were K-free van Harreveld solution and a similar solution with 20 mM-K.

constant amplitude from the resting potential to values of membrane potential 50 mV higher. It declined on depolarization. The fact that the excitatory potential did not increase with membrane potential, contrary to expectation on analogy with the end-plate potential in the frog (Fatt & Katz, 1951), and to what has been described qualitatively to occur in the crab (Fatt & Katz, 1953*b*) can be accounted for by a gradual decline in membrane resistance with increasing membrane potential. On the other hand, the noticeable bend in the inhibitory potential–membrane potential curve around the reversal potential must be attributed to a non-linearity of the conductance added during inhibitory transmitter action. This may well be present under other conditions, though it would usually be indistinguishable owing to the steep variation in the conductance of the non-junctional membrane.

The approximate reversal of the effect of high K is shown in Fig. 5. After bathing in 20 mM-K the muscle was washed and soaked for about 20 min in K-free solution before the fibre returned to the condition indicated in the final set of measurements. Similar difficulty in the reversal of the effect of high K was found in all other experiments.

Fig. 6 illustrates the effects of both Cl removal and K addition, including the addition of K to a Cl-free solution. The results for the removal of Cl from a K-free solution and the addition of K to a Cl-containing solution are similar to those already described. The addition of K to the Cl-free solution caused a fall in resting potential of 18 mV. In neither of the Cl-free solutions was the inhibitory potential made to reverse from a depolarization. However, from the trend of the curves at low membrane potentials it seems probable that the addition of K caused a shift of the reversal potential to a lower level of membrane potential. It was incidentally observed that the membrane resistance was not lowered to as great an extent by high K in the absence of Cl as in its presence. It was furthermore noted that a more rapid and complete reversal of the effect of high K was obtained if Cl were absent than if it were present. This may be accounted for by the fact that in the former case Cl would enter the fibre when the membrane resting potential was low.

Long-term effects

An attempt was made to observe effects caused by changes in the ionic composition of the interior of the muscle fibre, as distinct from the major changes described above, which are explicable in terms of alterations in the external solution with the internal composition approximately constant. Experiments involving observation of the properties of a fibre during extended soaking in Cl-free solution were not entirely successful, owing to frequent blockage of nerve conduction in this solution. Soaking for 12 min in Cl-free solution did not produce an obvious change (Fig. 4). The reverse experiment of returning the muscle to a Cl-containing solution after prolonged soaking in

Cl-free solution was more successful. The first set of measurements in Fig. 7 was made after soaking for 50 min in Cl-free solution. In view of the small size of the inhibitory potential recorded under these conditions, compared with that recorded later in the presence of Cl, it is probable that the amplitude of the response had declined during the prolonged soaking in Cl-free solution. As is shown by the second set of measurements, within 2 min of placing the muscle in a Cl-containing solution the resting potential rose 12·5 mV and the inhibitory potential reversed, to appear as a hyperpolarization at resting level. On displacing the membrane potential it now appeared that the response could be only slightly reversed beyond about 15 mV above the resting potential. After an interval of 20 min, during which the resting potential declined 1 mV, the

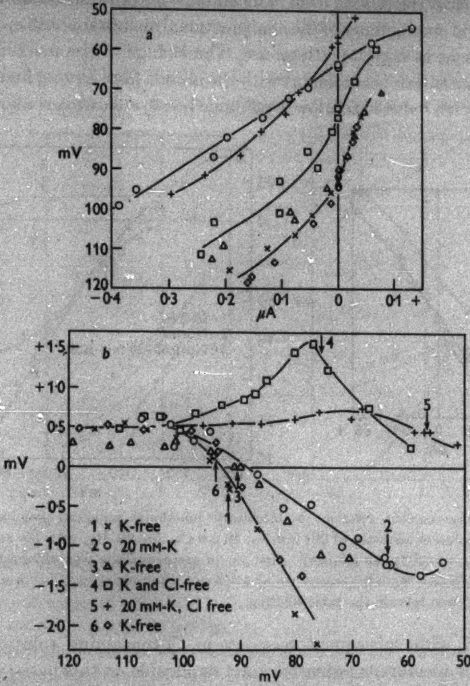

Fig. 6. *a*, Voltage–current relation; *b*, inhibitory junctional potential–membrane potential relation. Solutions were K-free van Harreveld solution, and a similar solution with 20 mM-K, a K- and Cl-free solution with pyroglutamate replacing Cl, and a similar Cl-free solution with 20 mM-K. Third set of measurements is not fitted by a curve.

reversal occurred about 6 mV above the resting potential and fairly large inhibitory potentials in the form of transient depolarization could be elicited at higher membrane potentials. The continued soaking also caused an increase in the response (in the form of a hyperpolarization) that was obtained at membrane potentials below the resting level. The voltage–current curves do not indicate any changes in conductance of the non-junctional membrane that might account for the changes in inhibitory response. The change in the behaviour at high membrane potentials is consistent with the muscle fibre having had its internal Cl concentration reduced to a low level immediately following soaking in Cl-free solution and with subsequent recovery in Cl-containing solution, but the change at low membrane potential is not explicable on this basis. One is thus brought to consider the possibility that prolonged soaking in Cl-free solution had affected the pre-junctional nerve terminals, causing a reduction in transmitter action from which there was a slow recovery after returning to Cl solution. A possible effect of the bathing solution on the nerve terminals does not enter into the interpretation of the other results that have been reported, which have been either on the reversal potential or on the shape of the inhibitory potential–membrane potential curve, rather than on the amplitude of the inhibitory potential. Following soaking in solutions of high K with Cl, there were the long-term changes already described which have been attributed to alterations in the internal Cl concentration.

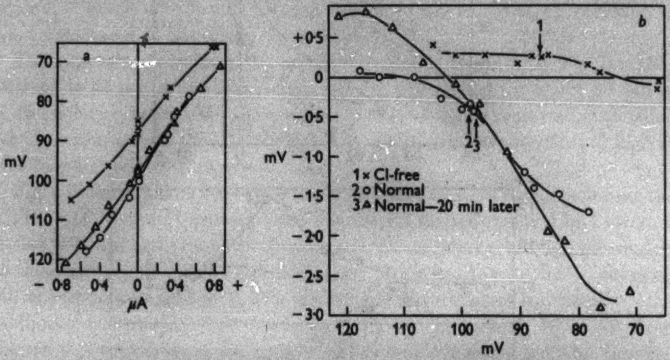

Fig. 7. *a*, Voltage–current relation; *b*, inhibitory junctional potential–membrane potential relation. Solution consisted of 201 mM-Na, 28 mM-Ca, 2·8 mM-Mg, with the anions either Cl (normal) or acetylglycine (Cl-free). First set of measurements was obtained after 50 min soaking in Cl-free solution; second set about 2 min after admitting Cl-containing solution; third set 20 min later in the same solution.

Response to γ-aminobutyric acid

There is evidence that this substance increases membrane conductance in various situations where an inhibitory transmitter produces a similar change (crayfish stretch receptor cell, S. W. Kuffler & C. Edwards, unpublished; crayfish muscle, B. L. Ginsborg, unpublished; third root giant motor axon of crayfish, E. Furshpan & D. Potter, unpublished). Voltage–current curves were taken in solutions of varying K and Cl content with and without γ-aminobutyric acid. Progressive changes in the voltage–current curves in a given solution were noticed, and measurements made in Cl-free or high K solutions were compared with the average of measurements made before and after in normal solution. In the experiments of Figs. 8 and 9, γ-aminobutyric acid was used in a concentration of 0·1 mM (about 10^{-5}, w/v), and observations in this were bracketed between observations in solutions without this substance.

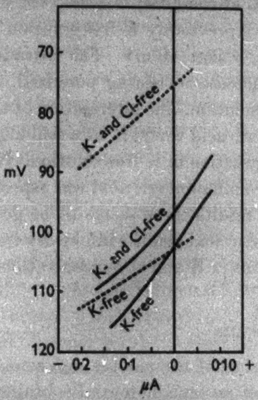

Fig. 8

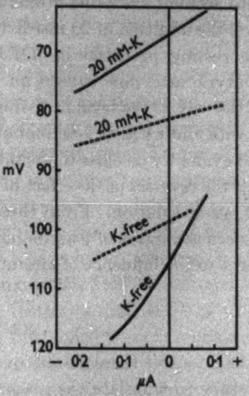

Fig. 9

Fig. 8. Averaged voltage–current curves obtained in a muscle fibre in absence of 0·1 mM-γ-aminobutyric acid (solid line) and in its presence (dotted line). Observations were made in a solution with normal Cl content and in a solution with Cl replaced by acetylglycine. Cations were 201 mM-Na, 28 mM-Ca, 2·8 mM-Mg.

Fig. 9. Averaged voltage–current curves obtained in a muscle fibre in absence of 0·1 mM-γ-aminobutyric acid (solid line) and in its presence (dotted line). Observations were made in K-free van Harreveld solution and in a similar solution with 20 mM-K.

In the normal (K-free) solution γ-aminobutyric acid caused a decrease in the slope of the curve without much change in resting potential (the fall of 6·5 mV in Fig. 9 was unusual). In K- and Cl-free solution (Fig. 8) the addition of γ-aminobutyric acid caused a decrease in slope associated with a considerable fall in resting potential. The equivalent quantity to the reversal potential, determined for inhibitory transmitter action, is the membrane potential at which the curves with and without γ-aminobutyric acid intersect. In the absence of Cl this was evidently several tens of millivolts below the already low resting potential occurring in the presence of the substance. With a constant concentration of γ-aminobutyric acid the fractional change in slope was significantly smaller in a Cl-free solution than in one containing Cl (Fig. 8). Calculating the action of γ-aminobutyric acid as an added conductance, in the Cl-free solution this was reduced to 0·4 times what it was in the Cl-containing solution (or about 0·2 times, when referred to the membrane conductance by taking into account the cable properties of the fibre).

In Fig. 9 the addition of 20 mM-K to a previously K-free solution is seen to cause the resting potential to fall by about 40 mV. The introduction of γ-aminobutyric acid now caused an increase in resting potential, returning it part of the way to the level in K-free solution. The intersection of the curves obtained with and without γ-aminobutyric acid would be at a still higher level, and would evidently be close to the intersection in K-free solution. It was also found that the reversal of the effect of γ-aminobutyric acid was especially slow in the 20 mM-K solution. From these results there seems to be good agreement between the action of γ-aminobutyric acid and the inhibitory transmitter with respect to the influence of alterations in K and Cl concentrations.

DISCUSSION

In order to interpret these results in terms of the passive movement of ions, it is necessary to translate the observed reversal potentials obtained during the application of current into corresponding equilibrium potentials (Burke & Ginsborg, 1956). Qualitatively, considerable use may be made of the fact that the equilibrium potential will lie between the resting potential and the reversal potential. The large shift of the equilibrium potential in the direction of lower membrane potential, which occurred on the removal of Cl from the bathing solution, is a clear indication that inhibitory action involves an increase in membrane permeability toward Cl. Thus, the response in the form of a depolarization which was observed in the Cl-free solution would be produced by an outward movement of Cl. The fact that the inhibitory potential could still be reversed at a membrane potential below the resting level can be accounted for by an accumulation of Cl in the solution outside the muscle fibre as a result of a steady loss of Cl from the fibre.

Increasing the K concentration caused a loss of membrane potential, as would be expected if the conductance of the resting membrane were dependent largely on the ability of K to move across the membrane. (The loss that occurred on the removal of Cl indicates that this ion also contributes to the conductance of the non-junctional membrane, but to a smaller extent.) The equilibrium potential for inhibition was not lowered as much as the resting potential by an increase in K concentration. What loss did occur in the level of the equilibrium potential can probably be explained by an influx of Cl at the reduced resting potential (accentuated possibly by a raised membrane permeability), with a resulting rise in internal Cl concentration. This interpretation is supported by the slow reversibility of the effect.

All the results are consistent with a passive movement of Cl playing a major role in the production of the inhibitory junctional potential, when the muscle was in a solution of normal ions. The possibility cannot be eliminated, however, that there may also be an increase in membrane permeability towards K. From the difference between the behaviour of the resting potential and the equilibrium potential on varying the concentrations of K and Cl it can be concluded that as far as K is concerned its partial conductance (or transport number) in the region of membrane responsible for producing the inhibitory response is small compared to that in the non-junctional membrane.

The conclusion reached here on the apparent insignificance of a K component in the conductance added during inhibitory transmitter action is in disagreement with the results of some other studies on inhibitory action. For cardiac muscle in vertebrates there is evidence from electrical measurements that acetylcholine exerts its inhibitory action by an increase in membrane permeability toward K (Burgen & Terroux, 1953). This conclusion is supported by observations of E. J. Harris & O. F. Hutter (unpublished) of an increased movement of tracer K; at the same time they failed to find an increased movement of tracer Cl. There can be little doubt that the mechanism of permeability change effected by acetylcholine on cardiac muscle is fundamentally different from that effected during inhibitory transmission in crayfish muscle. In connexion with closely related material, Fatt & Katz (1953*b*) reported briefly that transferring crab muscle from a solution with normal K concentration to a K-free one caused an increase in reversal potential exceeding the increase in resting potential. This is contrary to the present findings. It should be noted, however, that in the earlier experiment the problem of keeping the electrodes inserted while the solution was changed had not been solved, and it is possible that the result that was then obtained was influenced by injury which would cause a reduction in the resting potential while the equilibrium potential would be relatively unaffected. In any case the changes effected in the properties of a fibre by such a change of solution have in general been irreversible and are therefore less readily interpretable in terms of the change in external concentration of ions. More recently S. W. Kuffler & C. Edwards (unpublished) have found that the withdrawal of K (from normal van Harreveld solution) caused the response of the crayfish stretch receptor cell to inhibitory nerve fibre stimulation to alter in the direction of hyperpolarization, from which it is inferred that inhibitory nerve stimulation increases the partial conductance of the membrane to K. Possibly the stretch receptor nerve cell and the muscle fibre of the same animal respond differently during inhibitory transmitter action. (Note that the inhibitory nerve fibre supplying the stretch receptor cell also supplies some muscle fibres.) The difference may be quantitative, an increase in permeability toward K and Cl occurring in both cases, with the relative importance of the components of conductance contributed by the ion species depending on the post-junctional structure.

The mechanism of an increase in membrane permeability toward Cl (and probably other small anions) can be visualized as depending on the creation of charged pores. For the crayfish muscle fibre in its normal environment it appears that the equilibrium potential for inhibition coincides with the resting potential, and there is no reason to postulate a special mechanism (such as the metabolically-operated pumps described below) to act on the ions concerned in the inhibitory conductance increase. In the spinal motoneurone of the cat, however, a potential change in the form of a transient hyperpolarization occurs during inhibitory action, even though the membrane potential is at the resting level (Brock, Coombs & Eccles 1952). Inhibitory action involves an increase in membrane conductance with an equilibrium potential usually about 10 mV above the resting potential, and there is strong evidence for an increase in permeability toward Cl (Coombs *et al.* 1955). If in the motoneurone the conductance change is a result of an increase in permeability toward K as well as Cl, then it is possible to explain the failure of the equilibrium and resting potentials to coincide on the basis of an inward pumping of K, utilizing the free energy of metabolic reactions to raise the internal concentration above that which would be in equilibrium with the outside; it has been suggested that a pump of this sort exists in some other tissues. If, on the other hand, the conductance increase during inhibitory action involves mainly Cl, as has been found for crayfish muscle, the existence of a Cl pump extruding Cl from the cell must be postulated. The question might be raised whether the observed resting potential in the motoneurone is reduced by injury from a value 10 mV higher, corresponding to the equilibrium potential. The answer to this is to be found in the fact that the exchange of Cl between the inside and outside of the motoneurone following an intracellular injection of ions has a half-time of about 15 sec (Coombs *et al.* 1955), so that in the absence of a Cl pump this ion species would rapidly come into equilibrium.

SUMMARY

1. An intracellular electrode was used to record potential changes produced in a crayfish limb muscle on stimulation of its inhibitory nerve fibre. Observations were made over a range of membrane potentials, steady displacements being produced by current applied through a second intracellular electrode.

2. In the normal bathing solution the amplitude and sense of the inhibitory junctional potential varied with membrane potential, being directed toward a reversal potential which coincided approximately with the resting potential. The removal of K caused a rise in resting potential, together with a smaller rise in reversal potential. The effect was generally irreversible.

3. The removal of Cl (replacing it by organic anions) caused a fall in resting potential of 10–20 mV. At the resting potential in Cl-free solution the inhibitory response was in the form of a depolarization, the reversal potential occurring at a considerably lower membrane potential. The effect was immediately reversed on readmitting Cl.

4. Raising the K concentration from 0 to 20 mM with Cl present caused a fall in resting potential of about 30 mV. The reversal potential was nearly unchanged, being well above the resting potential in 20 mM-K. (The equilibrium potential would have changed by an intermediate amount.) The effect was only slowly and incompletely reversed.

5. Long-term alterations were observed which could be interpreted in terms of changes in the internal Cl concentration.

6. The action of γ-aminobutyric acid in increasing the membrane conductance was affected in a similar way by changes in K and Cl concentration as the response to inhibitory nerve fibre stimulation.

7. The action of inhibitory nerve fibre stimulation can be interpreted as a conductance increase depending on an increased permeability toward Cl.

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RESPONSES OF ISOLATED BRONCHIAL MUSCLE TO GANGLIONICALLY ACTIVE DRUGS

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It is generally accepted that the efferent innervation of the bronchial musculature is by branches from the vagus nerve and from the thoracic sympathetic trunk, entering the lung at its hilum (Macklin, 1929). The trachea is thought to have a similar supply. The existence of ganglion cells in the lung, distributed along the branches of the bronchial tree, has been known since the time of Remak (1844). The autonomic innervation appears to follow the usual pattern, in that the vagus fibres entering the lung are preganglionic and form synapses with Remak's ganglia within the lung; processes from these cells end on the smooth muscle cells in the walls of the bronchi. The sympathetic supply consists of post-ganglionic fibres which end directly on the smooth muscle of the bronchi.

Although it is usually held that the vagus nerve mediates bronchoconstriction and the sympathetic supply bronchodilatation, there is evidence that this simple picture is not completely correct. For instance, some workers have observed that under certain conditions excitation of the vagus, instead of producing the usual bronchoconstriction, may elicit bronchodilatation. There is little doubt that some of the earlier workers in fact stimulated not the vagus alone, but the vagosympathetic trunk. Further differences of opinion may have arisen from failure to realize the difficulty of demonstrating bronchodilatation in a preparation which has little tone under the experimental conditions. Dixon & Brodie (1903), who were aware of both these difficulties, concluded that the vagus nerve itself contained both bronchodilator and bronchoconstrictor fibres. This conclusion has subsequently been confirmed in guinea-pigs, cats, dogs and rabbits, the bronchodilator action of the vagus being more readily demonstrable after a dose of some bronchoconstrictor drug. Since the vagal bronchomotor fibres have been shown to end on the ganglion cells of the lung (Larsell & Mason, 1921) the question arises whether these ganglion cells play a part in mediating both types of vagal action, or whether their function is solely bronchoconstrictor.

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The present work was designed to study the characteristics of the ganglionic tissue within the trachea and large bronchi, using isolated preparations of trachea and bronchi from guinea-pig and cat. Pharmacological evidence will be presented that at least two types of ganglion cell, adrenergic and cholinergic, are present in these tissues.

METHODS

Material. Guinea-pig tracheae and bronchi were obtained from animals freshly stunned and bled out. Cat trachea was taken from animals which had been anaesthetized with ether for other purposes; large bronchi from these animals were taken at the end of experiments under ether or chloralose anaesthesia, lasting for 2–3 hr. We are grateful to Professor R. S. Pilcher for two human lungs, each removed at pneumonectomy for carcinoma in one lobe.

Isolated preparations. Chains of rings cut from bronchi and trachea, of the type described by Epstein (1932) and Castillo & de Beer (1947) were employed. Bronchi from guinea-pigs, cats, and humans were dissected free from lung tissue, and tied in chains with loops of cotton. Rings cut from guinea-pig trachea were orientated alternately and tied tightly with cotton so that the muscle strips were aligned. With cat trachea and the larger human bronchi single rings were often adequate. Rings cut by sectioning the trachea at intervals of 8–10 mm, containing two or three cartilaginous rings, were sometimes used; these were suspended by means of stitches put into the walls with a small curved needle.

The preparations were suspended in isolated organ baths, and the tonus level recorded with light balsa-wood frontal-writing levers recording on a lightly smoked drum, rotating at 1 mm/min. The solution used was Krebs–Henseleit solution: composition (g/l.), NaCl 6·87, KCl 0·42, $CaCl_2$ 0·28, $MgSO_4.7H_2O$ 0·29, $NaH_2PO_4.2H_2O$ 0·18, $NaHCO_3$ 2·10, glucose 1·00, aerated with oxygen and 5% CO_2 at 37° C. The bath volume was 50 ml.

The preparations were usually set up within 1 hr of removal from the animal, and then left for a further hour before testing. With guinea-pig trachea chains a marked rise in tone occurred during this time. The preparations responded to drugs for 10–12 hr. A special difficulty sometimes arose from the relatively high tone of the preparation of guinea-pig tracheal muscle. Not infrequently, washing out the bath led to a considerable change of tone, commonly a reduction, followed by a return to normal over a period of 10–20 min. In the absence of washing out only slow drifts of tone occurred, and there was no difficulty in distinguishing them from bronchoconstrictor or bronchodilator responses.

The Ringer's solution used by Epstein (1932) proved to be unsatisfactory. Preparations of guinea-pig trachea immersed in it were insensitive and soon ceased to respond to drugs at all.

Drugs. Drug concentrations were expressed in terms of the whole salt unless the contrary is stated. For the pA_2 determinations molar concentrations were computed in terms of $C_{33}H_{37}O_5N_5$, CH_3SO_3H for dihydroergotamine methanesulphonate and of $C_{33}H_{35}O_5N_5.\frac{1}{2}(C_4H_6O_6)$ for ergotamine tartrate.

The drugs used were: acetylcholine iodide, anagyrine hydriodide, ascorbic acid, atropine sulphate, barium chloride, B_1-pyrimidine hydrochloride (2-methyl-4-amino-5-methylamine pyrimidine; Roche Products), choline chloride, coniine hydrochloride, cytisine perchlorate, dibenamine hydrochloride, dibenzyline hydrochloride (Smith, Kline and French Laboratories, Ltd.), dihydroergotamine methanesulphonate, egg albumin (flake), ergometrine maleate, ergotamine tartrate, ergotoxine ethanesulphonate, hexamethonium bromide, histamine dihydrogen phosphate, L-adrenaline base, L-noradrenaline bitartrate monohydrate (Bayer Products, Ltd.), lobeline hydrochloride (Sandoz Products), mepyramine acid maleate (May and Baker, Ltd.), nicotine acid tartrate, phentolamine hydrochloride (7337, Rogitine; Ciba Laboratories), pilocarpine nitrate, physostigmine sulphate, prosympal (883F, diethylamino-methylbenzodioxane hydrochloride; May and Baker, Ltd.), sparteine sulphate, tetramethylammonium bromide, tolazoline hydrochloride (Priscol; Ciba Laboratories).

Nicotine acid tartrate was made up 1% or 10% (w/v), in the Krebs-Henseleit solution. Equivalent doses of tartaric acid were totally inactive under the experimental conditions. Adrenaline was dissolved in equivalent dilute HCl, and dilutions prepared in distilled water containing ascorbic acid 10^{-5}; noradrenaline was made up in ascorbic acid 10^{-6}. Fresh solutions of these drugs were prepared every 4–6 hr. Lobeline hydrochloride was dissolved at 1% in distilled water with the aid of a drop of dilute HCl. Dibenzyline was made up in 0·01 N-HCl. Ergotamine, dihydroergotamine, ergometrine, phentolamine and dibenamine were diluted from ampoules. Solutions of the remaining drugs were prepared in distilled water. The ergotoxine ethanesulphonate used was a routine production batch (Burroughs Wellcome), satisfied the B.P. requirements, and was estimated polarimetrically to contain 85% ergocornine, the remainder probably representing ergocristine.

We are grateful to the drug firms mentioned in the above list for supplies of the pure drugs.

RESULTS

Bronchodilator response of guinea-pig trachea, to nicotine

The principal action of nicotine on the guinea-pig tracheal chain is to produce a transient relaxation of the muscle. This response is obtained with concentrations of nicotine between 20 and 200 μg/ml. and is illustrated in Fig. 1.

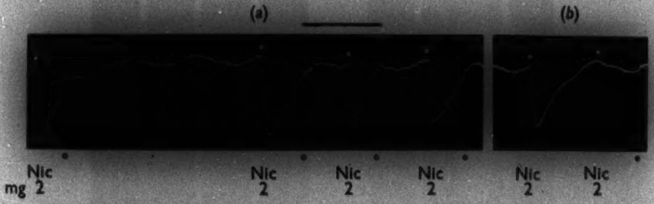

Fig. 1. Guinea-pig tracheal chain. In all figures, white dot marks the time of injection; ● denotes beginning of washing out; the black line at top of tracing gives 30 min time scale; bath capacity is 50 ml. (*a*) Successive responses to nicotin 2 mg added to bath; (*b*) 50 min later, abolition of nicotine response by leaving a previous dose in bath.

The response consists of an initial rapid relaxation followed by a slow return to the resting base line. If the nicotine is kept in the bath, the tracheal muscle is unresponsive to a further similar dose of nicotine (Fig. 1*b*). If the bath is washed out repeatedly, the relaxation response to nicotine can be repeated after an interval of 30–45 min. Thus with regular injections every 40 min, leaving the nicotine in contact for about 5 min, repeated constant responses to constant doses of nicotine or graded responses to graded doses may be obtained.

The manner in which the tissue responded rapidly to nicotine, then recovered from it, and finally became for a fairly prolonged period resistant to further doses of nicotine, strongly recalled the responses of ganglion cells to this drug.

We therefore proceeded to test further the possibility that there might be situated in the tissue ganglion cells possessing a bronchodilator action.

Hexamethonium. The response to nicotine was reduced by hexamethonium when present in a concentration of 0·1 μg/ml. (Fig. 2*a*). Such low concentrations of hexamethonium rarely show any action other than that of ganglion paralysis. The antagonism of the nicotine relaxation by these concentrations of hexamethonium therefore increased the likelihood that the nicotine action is

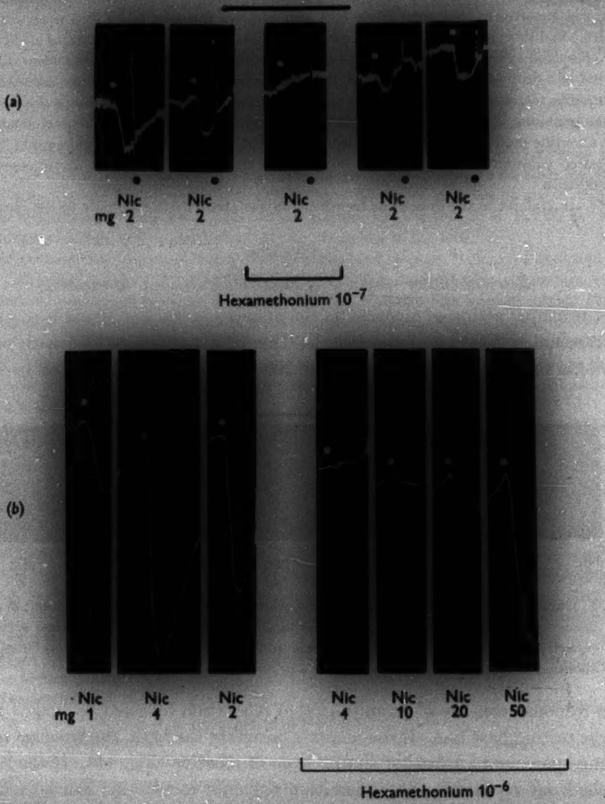

Fig. 2. Guinea-pig tracheal chain. (*a*) Effect of hexamethonium 10^{-7} on responses to 2 mg nicotine at 45 min intervals, hexamethonium added to bath 30 min before third dose of nicotine; (*b*) effect of hexamethonium 10^{-6} on various doses of nicotine, showing approximately 20-fold antagonism.

produced by excitation of bronchodilator ganglion cells situated in close relation to the tracheal muscle.

The antagonism between hexamethonium and nicotine appeared to be competitive, progressively higher concentrations of hexamethonium blocking responses to progressively larger doses of nicotine. For example, in one experiment hexamethonium 0·1 μg/ml. abolished responses to nicotine 50 μg/ml. but merely reduced the responses to nicotine 100 and 200 μg/ml. On the other hand, in the experiment shown in Fig. 2*b* a large dose of hexamethonium, 1·0 μg/ml., abolished responses to nicotine 40, 80 and 200 μg/ml, whereas nicotine 400 and 1000 μg/ml. still had an effect.

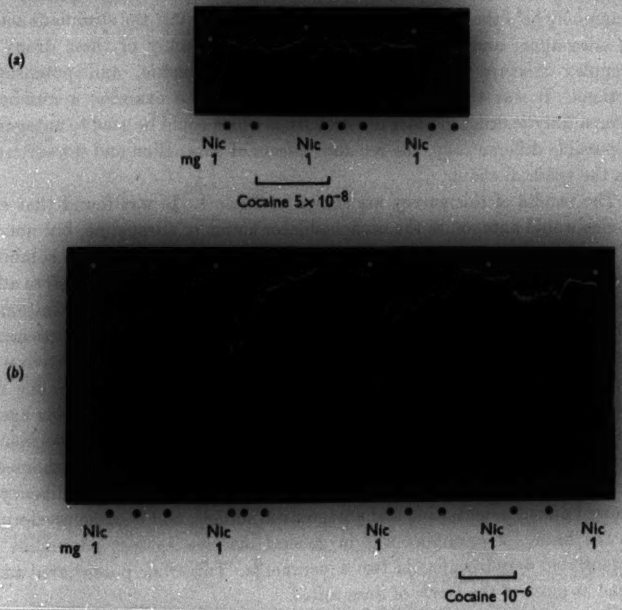

Fig. 3. Guinea-pig tracheal chain. (*a*) Effect of cocaine 5×10^{-8} on response to nicotine; (*b*) effect of cocaine 10^{-6} on response to nicotine.

Cocaine. If the response to nicotine is mediated by nervous tissue, then it should be reduced or abolished by local anaesthetic drugs. Figure 3 shows the effect of cocaine on the response; the nicotine response was reduced by a low concentration of cocaine (0·05 μg/ml.) and abolished by cocaine in a concentration of 1·0 μg/ml.

Nicotinic alkaloids. We have already mentioned that nicotine tends to block its own action on the tracheal muscle (Fig. 1*b*). It is known that related alkaloids can antagonize other responses to nicotine (Dale & Laidlaw, 1911–12) and the action of a number of these alkaloids was examined. It was found that lobeline 1 μg/ml., coniine 0·5 μg/ml., cytisine 5 μg/ml., sparteine 0·5 μg/ml. and anagyrine 2 μg/ml. all abolished the nicotine response. These alkaloids did not affect bronchodilator responses to adrenaline or noradrenaline.

Sympatholytic drugs. Since the response to nicotine was bronchodilator, the ganglion cells concerned might be supposed to mediate the response by liberating sympathin at their post-ganglionic endings. Some difficulty was expected in testing this conclusion with sympatholytic drugs, for two reasons. It is commonly held that such drugs are only effective against the stimulant actions of adrenaline; and the pharmacological effect of many of these drugs is a complex mixture of sympatholytic, sympathomimetic, and potentiating actions. It was therefore judged necessary first to examine a number of sympatholytic drugs in order to select drugs which could be used to antagonize, if possible differentially, the relaxant effects of adrenaline and noradrenaline on the tracheal chain.

The results of this survey are shown in Table 1. It was found that ergotoxine would antagonize the bronchodilator action of adrenaline, but not that of noradrenaline. This drug is unsatisfactory to use because of its low solubility; it tends to be precipitated after addition to the bath. It was therefore added to the bath as a fine aqueous suspension to give a final 'concentration' of 100 μg/ml.; under these conditions the drug was precipitated, but presumably gave a saturated solution. Ergotoxine was unsuitable for quantitative work, but ergotamine and dihydroergotamine both antagonized adrenaline over fairly wide ranges of concentration. The results with these alkaloids against noradrenaline were rather irregular. In a single test, ergometrine was inactive against both amines. Prosympal (883 F) and phentolamine were both inactive against the amines in the concentrations tested. Dibenamine and dibenzyline showed mixed potentiating and antagonistic actions, and became precipitated in the bath. Mepyramine was in general inactive against adrenaline but potentiated noradrenaline in two experiments. Tolazoline potentiated adrenaline at most of the levels of dose tested.

Ergotoxine used as just described, to give a concentration in the bath of something less than 100 μg/ml., abolished the bronchodilator response of the guinea-pig trachea to nicotine, and also the dilator response to adrenaline, leaving the response to noradrenaline unaffected (Fig. 4*a*). This indicated that the nerve endings mediating the nicotine response were adrenergic, and suggested that the transmitter was adrenaline itself.

In a number of experiments the actions of low concentrations of dihydroergotamine and ergotamine on responses to adrenaline and nicotine were

TABLE 1. Antagonism of bronchodilator actions of adrenaline and noradrenaline by sympatholytic drugs. Summary of experiments on guinea-pig trachea

Antagonist	Concentration	Action against: Adrenaline	Action against: Noradrenaline	
Dibenamine	10^{-5}	0	0	Potentiated adrenaline and norad.
	10^{-4}	0	.	Precipitated; potentiated
	10^{-3}	+	.	Precipitated
Dibenzyline	10^{-6}	0	0	Potentiated adrenaline and norad.
	10^{-5}	0	0	Potentiated adrenaline
	4×10^{-5}	+	.	Precipitated
Dihydroergotamine	10^{-8}	0	0	
	10^{-7}	0	+	.
	2×10^{-7}	+	0	.
	$3 \times 10^{-7} - 8 \times 10^{-7}$	+	.	.
	10^{-6}	+	+	.
	2×10^{-6}	+	.	.
	5×10^{-6}	+	0	.
	10^{-5}	.	0, +	.
	$1{\cdot}6 \times 10^{-5}$	.	.	.
Ergotamine	2×10^{-7}	0	.	.
	5×10^{-7}	0, +	.	.
	8×10^{-7}	+	.	.
	10^{-6}	+	0	.
	4×10^{-6}	.	+	.
	5×10^{-6}	+	.	.
	8×10^{-6}	.	0, +	.
	10^{-5}	+	0	Potentiated noradrenaline
	$1{\cdot}6 \times 10^{-5}$	.	0	.
	2×10^{-5}	.	+, +	.
Ergometrine	10^{-6}	0	0	.
Ergotoxine	10^{-5}	0	0	.
	10^{-4}	+ +	0	Precipitated
Mepyramine	$1{\cdot}6 \times 10^{-8}$	.	0	.
	8×10^{-8}	0	.	.
	$1{\cdot}6 \times 10^{-7}$	.	0	.
	8×10^{-7}	0, +	0	Potentiated noradrenaline
	8×10^{-6}	0	0	.
Phentolamine	2×10^{-6}	0	0	.
Prosympal	$10^{-6} - 10^{-5}$	0	0	.
	10^{-4}	0	.	.
Tolazoline	$10^{-6} - 2 \times 10^{-6}$	0	.	.
	$5 \times 10^{-6} - 2 \times 10^{-5}$	0	.	Potentiated
	5×10^{-5}	0	.	.
	10^{-4}	0	0	Potentiated adrenaline and norad.

+ = Antagonism; 0 = no antagonism.

compared. One such experiment is illustrated in Fig. 4*b*, where it will be seen that dihydroergotamine 0·2 μg/ml. barely affected the responses to adrenaline and nicotine, while in higher concentrations, 1·0 μg/ml., the antagonist reduced both responses to approximately the same extent. In general the effects of these ergot alkaloids in antagonizing the nicotine response paralleled their effects in antagonizing equivalent responses to adrenaline; considerably higher concentrations of antagonist were required to affect the response to noradrenaline materially.

The activities of the two most suitable antagonists, ergotamine and dihydroergotamine, against adrenaline, noradrenaline, and nicotine, were assessed quantitatively. In addition, since Lockett (1957) has produced evidence in the intact cat that isoprenaline mediates the responses of the bronchi to sympathetic stimulation, we also examined the antagonism of these sympatholytics to isoprenaline. The results are given in the pA_2 notation of Schild (1947*a*, *b*) which expresses the activity of an antagonist as the negative logarithm of the concentration required to reduce the response of a double dose of the active drug to the same level as the response of a single dose of active drug in the absence of antagonist. The ergot compounds caused a rise in base line, which prevented these measurements being fully accurate when the antagonist was

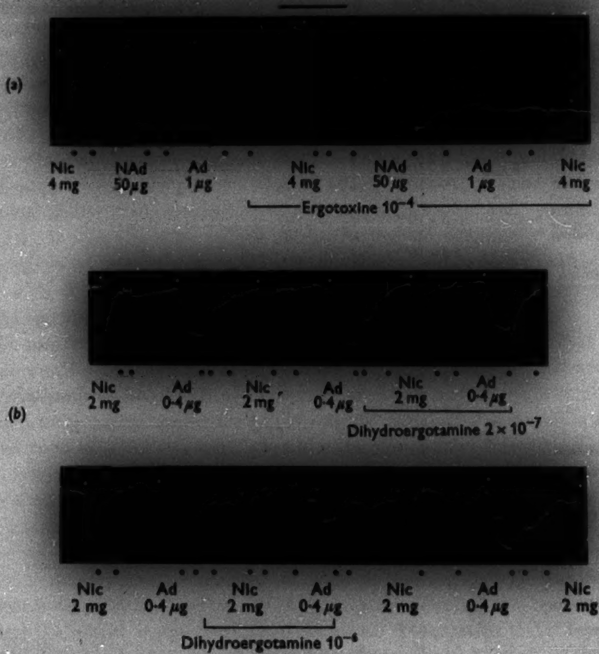

Fig. 4. Guinea-pig tracheal chain. (*a*) Action of ergotoxine 10^{-4}, abolishing response to nicotine and adrenaline but not that to noradrenaline; (*b*) action of dihydroergotamine 2×10^{-7} and 10^{-6} on responses to adrenaline and nicotine.

added. The figures for pA_2 given represent estimates obtained as in Fig. 5; this shows the effects of a concentration of antagonist definitely greater than the pA_2, of a concentration approximately equal to the pA_2, and of a concentration definitely less than the pA_2. In records where the base line was elevated by the ergot alkaloid, response heights were estimated from the base line immediately before addition of the dose of nicotine. Two series of determinations were performed; in the first the antagonist was allowed to remain in contact with the tissue for 20 min before the dose of active drug was given, whereas in the second series a 30 min contact was used. The results in the two series did not differ materially and have been combined in Fig. 6 (showing the individual observations), and in Table 2, which summarizes the pA_2 values deduced. With the antagonisms between the ergot compounds and noradrenaline the results were rather irregular, and potentiation sometimes occurred with the highest concentrations of antagonist. The values for these antagonisms in Table 2 are therefore shown in parentheses. The principal result to be deduced from Fig. 6, and Table 2, is that the absolute activities of the two antagonists against nicotine and against adrenaline or isoprenaline were of the same order, but they differed widely from the absolute activities against noradrenaline.

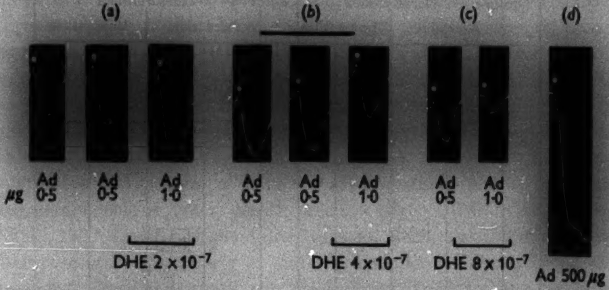

Fig. 5. Guinea-pig tracheal chain. Part of an estimation of pA_2 for dihydroergotamine against adrenaline. The last panel gives the effect of a large dose of adrenaline, showing that submaximal responses were used.

These quantitative experiments therefore confirm the results obtained with ergotoxine: (*a*) that the response to nicotine is mediated by a sympathomimetic amine; and (*b*) that the amine concerned resembles adrenaline rather than noradrenaline. Adrenaline, however, is not the only candidate; isoprenaline also has the pharmacological properties required.

Sympathetic potentiators. The association between local anaesthetic activity, anti-amine oxidase activity, and potentiation of sympathomimetic activity is well known. It was therefore expected that it would be difficult to demonstrate potentiation of the response to nicotine by known adrenaline potentiators. This proved to be the case. For example, with cocaine the lowest concentration with which we were able reproducibly to potentiate adrenaline responses on the trachea was about 100 μg/ml., whereas this local anaesthetic will often antagonize the nicotine response in concentrations as low as 0·02 μg/ml.

Rosa & McDowall (1951) reported that ascorbic acid increases the response of isolated human bronchial muscle to adrenaline. We were unable to confirm this on guinea-pig trachea, and the action of ascorbic acid on the nicotine response was difficult to assess, because irregular changes in the base line followed the addition of ascorbic acid to the bath.

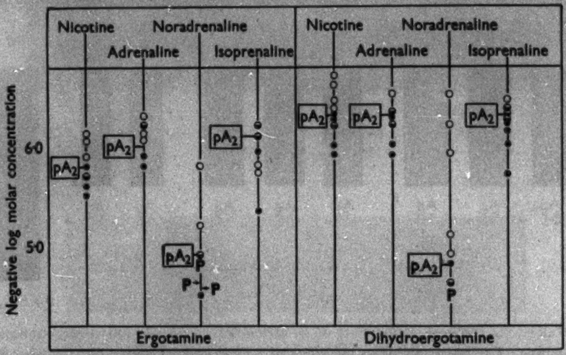

Fig. 6. Diagram of results of experiments to estimate pA_2 of ergotamine and dihydroergotamine against nicotine, adrenaline, noradrenaline, and isoprenaline. ○, $>pA_2$; ◒, $=pA_2$ approx.; ●, $<pA_2$; P, potentiation.

Table 2. pA_2 values and relative potencies of sympatholytics in the antagonism of bronchodilator effects on the guinea-pig trachea

		Nicotine	Adrenaline	Noradrenaline	Isoprenaline
pA_2	Ergotamine	5·8	6·0	(4·9)	6·1
	Dihydroergotamine	6·3	6·3	(4·8)	6·3
Relative potency	Ergotamine	60	100	(8)	130
	Dihydroergotamine	200	200	(6)	200

Ergotamine versus adrenaline = 100.

Other drugs. *Atropine* in concentrations from 1 to 100 μg/ml. reduced the bronchodilator nicotine response without affecting the response to adrenaline or noradrenaline. The minimum concentration of atropine found to be active in this respect was more than one hundred times that required to reduce an acetylcholine contraction of the trachea. *Mepyramine* had a rather irregular influence on the response to adrenaline and noradrenaline, sometimes potentiating the action of the latter. It reduced the nicotine response. *Tolazoline*, which regularly potentiated the action of adrenaline, had an anti-nicotinic action in concentrations of 10 and 20 μg/ml. *Prosympal*, which was inactive in concentrations up to 100 μg/ml. against the bronchodilator responses to noradrenaline and adrenaline, antagonized nicotine bronchodilatation at 1 and 2 μg/ml. *Phentolamine* was similarly inactive against the sympathetic amines at 2 μg/ml., but reduced the nicotine response.

The general pattern of action of this group of drugs, depression of the response to nicotine but not to sympathetic amines, raises one of the recurrent difficulties in work of this kind with imperfectly specific drugs. Many such drugs are able to produce local anaesthesia, a property common to a wide range of aromatic amines (Burn, 1956). Such an action, either by depressing conduction in post-ganglionic nerve fibres or by block at the ganglion synaptic area (Harvey, 1939) would lead to a reduction of the action of nicotine in our experiments, without necessarily directly affecting the response of the bronchial smooth muscle itself to drugs such as adrenaline. It is known that atropine, mepyramine and prosympal have a local anaesthetic action, the first two being comparable in potency with procaine (Bacq, 1935; Wierzuchowski & Bielinki, 1939; Dews & Graham, 1946; Dutta, 1949). Tolazoline and phentolamine possess a wide variety of actions (Goodman & Gilman, 1955), and although their local anaesthetic potency does not appear to have been studied, it would be surprising, from the general properties of such drugs, if they did not possess it in some degree. Since cocaine in a dilution of one in twenty million reduced the nicotine response, a potency one-twentieth of that of cocaine, or less, would suffice to account for the actions of these compounds. We have, therefore, attributed the results described to their local anaesthetic action on the nervous tissue in the bronchial muscle preparations.

Thus the bronchodilator response of the guinea-pig tracheal chain to nicotine possesses the pharmacological characteristics of an excitation of ganglion cells situated in the tissue, releasing adrenaline at their post-ganglionic endings. These characteristics may be summarized as follows:

(1) The tissue recovers from the effect of nicotine without removal of the drug from the bath and in this state is refractory to the addition of further doses of nicotine.

(2) The effect of nicotine is blocked by very low doses of hexamethonium and by 'nicotinic' alkaloids.

(3) The response is unaffected by specific concentrations of atropine.

(4) The response is reduced by ergotoxine, ergotamine and dihydroergotamine; the effects of these sympatholytic drugs parallel their effects on adrenaline and isoprenaline responses, but not their effects on noradrenaline responses.

(5) The response is abolished by drugs having a local anaesthetic action.

Bronchoconstrictor response of guinea-pig trachea to nicotine

It was expected at the outset that cholinergic ganglion cells on the trachea would give rise to a bronchoconstrictor response to nicotine. The most prominent response was the one we have just described, bronchodilatation. It was, however, noted that with fresh preparations the earliest responses were sometimes diphasic in character, relaxation of the smooth muscle being preceded by a brief and small contraction. Examples of this diphasic response are shown in Fig. 7*a*, *b*. A similar type of response was occasionally seen after the tone of the preparation had been temporarily reduced by some means. Fig. 7*c* shows a diphasic response to nicotine obtained during a reduction of tone persisting after a response to adrenaline. Fig. 7*d* shows a similar diphasic response produced by nicotine during a depression of tone after a response to acetylcholine.

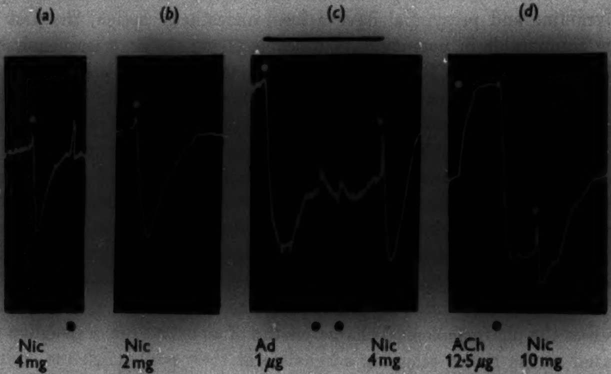

Fig. 7. Guinea-pig tracheal chain. Diphasic responses to nicotine obtained (*a*) and (*b*) spontaneously in two preparations; (*c*) and (*d*) during a persisting relaxation following adrenaline or acetylcholine washing out respectively.

This suggested that a number of cholinergic ganglion cells were responding to nicotine in our experiments, but that the response was greatly overshadowed by that of the adrenergic ganglion cells. We therefore examined the action of eserine, to see whether this drug would influence the response of the tracheal chain to nicotine. This showed at once the presence of a cholinergic response, the bronchoconstrictor component being greatly enhanced, so far as to cause an actual reversal of the response to nicotine (Fig. 8). This bronchoconstrictor response to nicotine resembled in its general pharmacological properties the bronchodilator response, in that it was antagonized by the presence of a previous dose of nicotine in the bath, by specific concentrations of hexamethonium and by specific concentrations of the appropriate antagonist of the transmitter, in this case atropine.

Other responses to nicotine and nicotinic drugs. An additional response by the tracheal chain to low doses of nicotine was observed in a number of experiments. When a preparation was tested within an hour or two of its being set up, using doses of nicotine between 1 and 10 μg/ml., it responded with a very small slow contraction, of the order of 5% of the maximal contraction to barium. This contraction could not be repeated more than once or, rarely, twice, and it was resistant both to hexamethonium in concentrations up to 400 μg/ml. and to atropine up to 10 μg/ml. This small contraction seemed to be somewhat more easily obtained when the tracheal chains were so cut that the individual rings were wider than usual and each included three or four cartilaginous rings. Since these responses are not antagonized by hexamethonium or atropine, it is considered that they are not typical ganglion-cell responses, nor muscarinic actions.

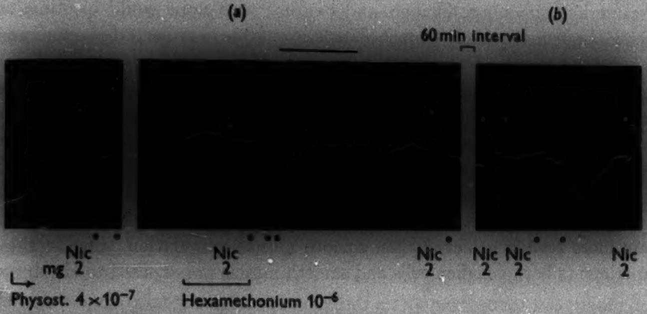

Fig. 8. Guinea-pig tracheal chain. Tone raised by physostigmine 4×10^{-7}. Effect of nicotine (*a*) before, during and after hexamethonium 10^{-6}; (*b*) in the presence of a previous dose of nicotine.

Other alkaloids related to nicotine also exhibited some anomalous effects. The principal actions of these alkaloids on smooth muscle are generally thought to be mediated by initial stimulation followed by secondary depression of autonomic ganglia. In our experiments lobeline, cytisine, coniine, anagyrine, and sparteine were found to give contractions of tracheal chains in moderate dose, and lobeline and anagyrine gave relaxations with higher doses. With all these drugs except lobeline the responses were relatively small. They were unlike the rapid transient nicotine responses and were resistant to hexamethonium. Further, the bronchoconstrictor responses were not antagonized by atropine, and the bronchodilator responses resisted dihydroergotamine. It was concluded that although these alkaloids bear a superficial resemblance to nicotine in their actions on certain smooth-muscle preparations, they do not in fact elicit the type of ganglion response given by nicotine on the tracheal chain.

The remaining drugs tested on guinea-pig trachea, which possess ganglion-stimulant properties in other situations such as the superior cervical ganglion (Burn & Dale, 1914–15; Feldberg & Vartiainen, 1935; Ambache, 1949), were tetramethylammonium, choline, and barium; with none could nicotinic responses be demonstrated. Bronchoconstrictions due to tetramethylammonium were not antagonized by hexamethonium (up to 500 μg/ml.), but were abolished by atropine 1 μg/ml. The bronchoconstrictor responses of the trachea to choline were unaffected by hexamethonium (up to 500 μg/ml.); those to barium were unaffected by hexamethonium, in concentrations of 1 and 10 μg/ml. It does not necessarily follow that these substances are incapable of nicotinic action on bronchial ganglion cells. Since they can all excite smooth muscle directly, ganglionic excitation would only show itself clearly if this were more readily achieved than smooth muscle stimulation, since in these experiments both ganglion cells and smooth muscle are exposed to the drugs.

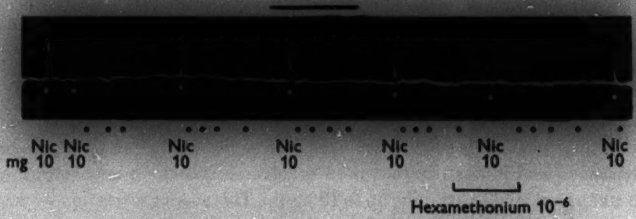

Fig. 9. Cat tracheal ring. Response to nicotine, normally, in the presence of a previous dose or in the presence of hexamethonium 10^{-6}.

Action of nicotine on cat trachea

Under our experimental conditions, cat tracheal rings, unlike guinea-pig rings, have no resting tone. Whereas guinea-pig rings are immediately relaxed by sympathomimetic amines and by drugs such as caffeine and papaverine, bronchodilator drugs produce no response on the cat trachea unless the preparation has previously been made to contract by some bronchoconstrictor drug.

It was therefore not surprising to find that the primary response of cat trachea to nicotine was not bronchodilatation but a contraction resembling in all respects that seen after physostigmine in the guinea-pig preparation. With the majority of cat tracheal rings nicotine in the range of concentrations

20–400 μg/ml. produced small transient contractions (Fig. 9). After the contraction the trachea was unresponsive to further doses of nicotine until the bath had been repeatedly washed out (Fig. 9). Repeatable and graded responses could be obtained at intervals of about 45 min.

Nature of the bronchoconstrictor response of cat trachea to nicotine. The bronchoconstrictor response was greatly potentiated by physostigmine 0·1 and 1 μg/ml. (Fig. 10*a*), and typical responses to nicotine occurred in those preparations which had previously been unresponsive (Fig. 10*b*). Hexamethonium 1 μg/ml., cocaine 1 μg/ml., or atropine 1 μg/ml. all antagonized the response. Fig. 10*b* shows also that these concentrations of hexamethonium were specific and had no atropine-like action, since the equivalent responses to acetylcholine were unaffected; atropine, as would be expected, blocked the actions of both nicotine and acetylcholine.

The bronchoconstrictor responses of the isolated cat's tracheal ring to nicotine have, therefore, the characteristics of a cholinergic ganglion-cell response, since they are antagonized: (1) by the presence of a previous dose of nicotine; (2) by hexamethonium; (3) by cocaine and (4) by atropine; but are (5) potentiated by eserine.

Bronchodilator response to nicotine. It seemed possible that the failure to observe bronchodilator responses to nicotine on the cat trachea was simply due to the absence of tone in this preparation. The response to nicotine was therefore re-examined on chains which were maintained in a partly contracted state by the presence of pilocarpine 0·25 μg/ml. It was found that under these conditions 20–50 μg/ml. of nicotine gave transient relaxations resembling closely the responses of the guinea-pig trachea to similar doses (Fig. 11). The response to nicotine was blocked by the presence of the previous dose of nicotine left in the bath and by hexamethonium 10 μg/ml. It therefore seemed likely that these responses were mediated by adrenergic ganglion cells, but the responses were not affected by ergotamine 5 μg/ml.

We have concluded that when the cat trachea is partly contracted its primary response to nicotine resembles in most respects that of the guinea-pig trachea. On both preparations the majority of ganglion cells responding to nicotine appear to be adrenergic, producing bronchodilatation, but the presence of cholinergic ganglion cells, producing a bronchoconstriction, can be demonstrated either directly or in the presence of physostigmine.

Action of nicotine on isolated guinea-pig primary bronchi

The preceding analysis of the responses of guinea-pig and cat trachea to nicotine provided the basis for interpretation of the more complicated response of isolated chain preparations made from the main bronchi of the guinea-pig. The responses of this preparation to nicotine 40–80 μg/ml. had three components. All three were greatly reduced or abolished by hexamethonium

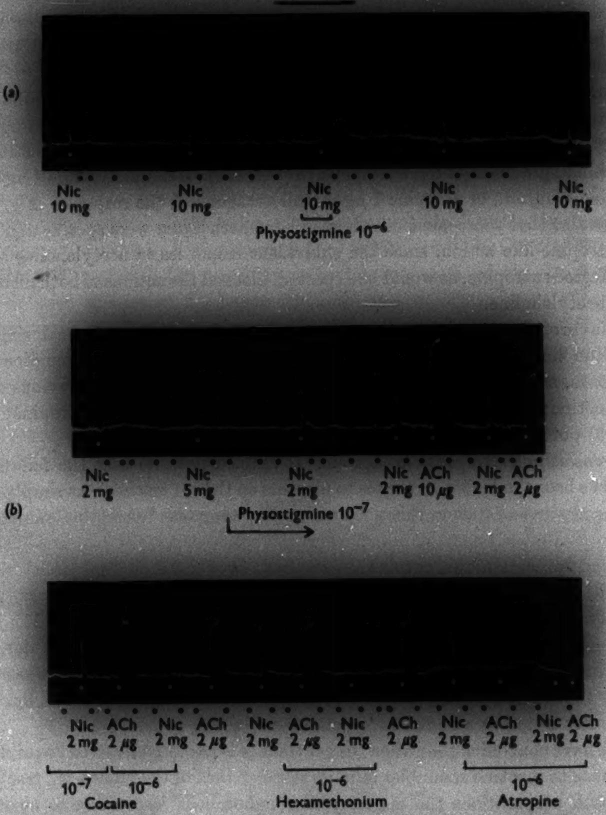

Fig. 10. Cat tracheal ring. (*a*) Action of nicotine before, during and after physostigmine 10^{-6}; (*b*) action of nicotine before and during exposure to physostigmine; abolition of nicotine action by cocaine and by hexamethonium, and of the actions of both nicotine and acetylcholine by atropine.

1 μg/ml., or by cocaine 1 μg/ml. and were therefore presumed to be due to the action of nicotine on ganglion cells in the tissue.

The first component was a rapid transient contraction readily abolished by atropine 0·2 or 1·0 μg/ml. (Fig. 12*a*, *b*), and potentiated by physostigmine, 0·1 μg/ml. (Fig. 12*c*). It therefore possessed the pharmacological properties of a cholinergic ganglion-cell response. This transient bronchoconstriction sometimes waned with repeated responses and then merged with the second component of the nicotine response.

The second component of the response of the guinea-pig's primary bronchus to nicotine consisted of a long-lasting bronchoconstriction, shown in Fig. 12*a*. This part of the response was unaffected by specific concentrations of atropine (Fig. 12*a*, *b*), though higher concentrations of atropine, up to 5 μg/ml., could reduce it somewhat. This is not surprising, since the effect was sensitive to ganglion-blocking drugs, and it is known that high concentrations of atropine have some ganglion-blocking activity. The relative resistance of the second component to atropine suggested that this part of the nicotine response was not cholinergic in nature. This was confirmed by finding that physostigmine, which potentiated the first component markedly, left the second component quite unaffected.

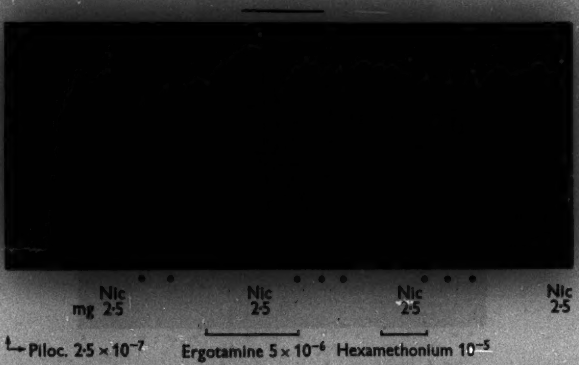

Fig. 11. Cat tracheal ring; tone raised by pilocarpine throughout. Bronchodilator action of nicotine, resistant to ergotamine but greatly reduced by hexamethonium.

Since the second component of the nicotine response appeared not to be cholinergic in nature, we tested the possibility that histamine might be the post-ganglionic mediator concerned. Figure 13*a* shows such an experiment. It will be seen that the nicotine response displayed the second component fairly well; alternate responses were to histamine. On introducing mepyramine into the Ringer's solution the nicotine response was quite unaffected, although the effects of histamine were abolished. It was found that much higher concentrations of mepyramine, such as might be expected to exhibit local anaesthetic action, would cause some reduction of the nicotine effect, but these concentrations were one thousand times greater than is necessary to reduce the effect of histamine. Further, the specific histamine potentiator B_1-pyrimidine

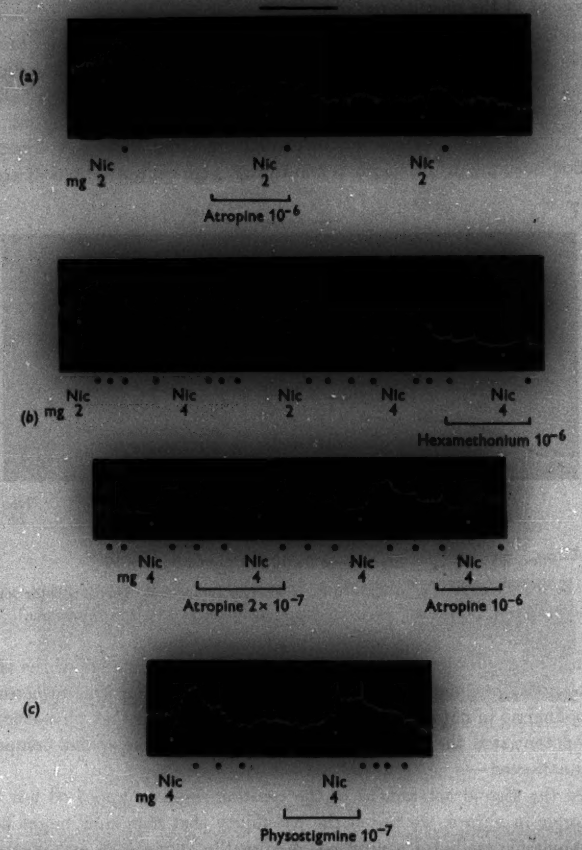

Fig. 12. Guinea-pig primary bronchus. (*a*) Response to nicotine, and abolition by atropine of initial bronchoconstriction only—after atropine is washed out initial bronchodilatation occurs; (*b*) response to nicotine, showing its abolition by hexamethonium and the resistance of the slow component of the response to atropine; (*c*) response to nicotine with initial bronchodilatation; action of physostigmine, showing reversal of the initial response without change of the slow response.

(Arunlakshana, Mongar & Schild, 1954) did not affect the second component of the nicotine response although it strongly potentiated an equivalent histamine response (Fig. 13*b*).

The remaining component of the response of guinea-pig primary bronchi to nicotine, a rapid transient relaxation, was only demonstrable after the first component had been removed by atropine (Figs. 11*a*, 12*a*, *c*). It was blocked by hexamethonium or cocaine. It was not analysed further, but so far as it was studied it resembled the bronchodilator response of the guinea-pig trachea to nicotine, or of the cat trachea after exposure to pilocarpine. This component therefore had the characteristics of a response of adrenergic ganglia.

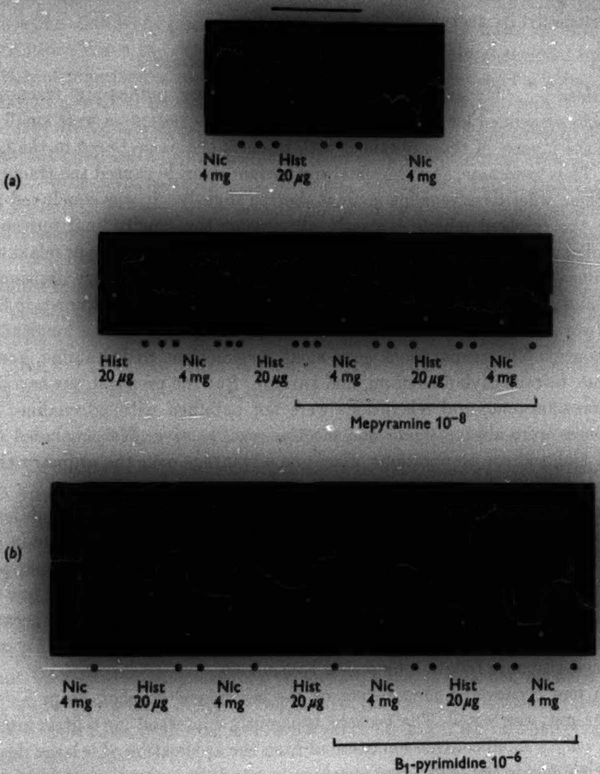

Fig. 13. Guinea-pig primary bronchi. Response to nicotine and to histamine, showing resistance of nicotine slow bronchoconstriction (*a*) to mepyramine, and (*b*) to B_1-pyrimidine.

Thus while the two rapid, transient components of the response to nicotine by the guinea-pig primary bronchus can be readily interpreted as responses mediated by cholinergic and adrenergic ganglion cells respectively, the prolonged bronchoconstrictor component of the response remains unexplained. This bronchoconstriction has the characteristics of a ganglion-cell response, since it is abolished by hexamethonium and cocaine; but it resists atropine, physostigmine, mepyramine, and B_1-pyrimidine, and is therefore presumably not mediated by acetylcholine or by histamine.

Other bronchial muscle preparations

Guinea-pig. Experiments were performed using guinea-pig trachea or bronchi suspended longitudinally. With trachea the responses were small and were the inverse of those obtained with tracheal chains prepared in the usual way. Thus histamine, acetylcholine, and barium chloride caused the trachea to lengthen, whereas adrenaline made it shorten slightly. It was concluded that since the tracheal muscle is nearly all circular the longitudinal movement recorded was merely a secondary effect of the circular contraction or relaxation.

Guinea-pig main bronchi, consisting of primary and secondary divisions in continuity, were also tested longitudinally. The muscle here is known to form a network, so that any contraction would be expected to have a longitudinal component. The preparations gave small contractions to histamine, acetylcholine, tetramethylammonium, and barium. But the preparations had little spontaneous tone, and relaxations were not obtained with adrenaline. The responses were always small, and nicotine was inactive. We concluded that these longitudinal preparations offered no advantage over the ordinary chain preparations, and they were not studied any further.

Experiments were also made on tracheal chains prepared from different parts of the trachea. It was found that nicotine was often less active in producing relaxation of the lower third of the trachea than of the upper part, whereas the sensitivity to the sympathomimetic amines was more or less uniform. The responses of the lower third of the trachea, indeed, on occasion seemed to resemble those of primary bronchial chains, consisting of a rapid diphasic response followed by a slow contracture.

On one occasion a preparation of the secondary bronchi, from a large guinea-pig, was tested. The bronchi apparently contained very little muscle, since only a small contraction resulted from the application of a large dose of acetylcholine to the bath. Responses to nicotine in this experiment were small and of doubtful significance.

Air-perfused guinea-pig lungs. A preparation of the type described by Arunlakshana & Schild (1950) was used, the pulmonary circulation being perfused with Krebs–Henseleit solution, the bronchial tree with a constant flow of air. The pressure developed in the trachea was recorded with a small mercury manometer, and provided a measure of the bronchial diameter. Under these conditions the bronchi have little or no tone and adrenaline was nactive. After the perfusion had been set up for 1–2 hr, nicotine injected into the pulmonary artery in a dose of 0·5–1 mg caused a transient bronchoconstriction, which was prevented by previously administered hexamethonium.

Cat primary and secondary bronchial chains. These preparations gave responses similar to those of the cat trachea. Nicotine was inactive on the resting preparation, but in the presence of physostigmine elicited a transient bronchoconstriction. When the preparation was maintained partly contracted by the addition of pilocarpine, nicotine produced a transient bronchodilatation. The relaxation produced by nicotine was prevented by hexamethonium.

Human bronchial chains. Six preparations were made from bronchi of the fourth, fifth and sixth orders, dissected from apparently normal lobes of lungs removed from two patients with bronchial carcinoma. These preparations have some resting tone; they can be stimulated by acetylcholine and relaxed by adrenaline or noradrenaline. The response to acetylcholine has been shown to be increased by physostigmine (Hawkins & Schild, 1951). No clear-cut responses to nicotine were obtained, however, even in the presence of eserine. It is in fact known that as the periphery of the human bronchial tree is approached ganglion cells rapidly become scanty, the post-ganglionic axons becoming progressively longer and more longitudinally placed and hence more likely to be severed in ring preparations.

Other actions of hexamethonium on bronchial muscle

The usefulness of hexamethonium in studying ganglionic physiology depends on its specificity of action. Although it has so far proved satisfactory in this respect, some additional experiments were made on bronchial muscle to test how far actions other than ganglionic might be present to complicate the interpretation of our experiments on this tissue.

It was noticed that large amounts of hexamethonium (200–800 μg/ml.) caused a slowly developing rise in tone of guinea-pig tracheal chains. This was a small but regular action, seen both with the bromide and iodide salts. It cannot be regarded as muscarinic in nature, since it was not abolished by atropine (1–10 μg/ml.); atropine could, however, delay the action somewhat. Large doses of hexamethonium had no effect of their own on cat bronchial muscle.

Hexamethonium concentrations up to 500 μg/ml. did not antagonize the responses of guinea-pig bronchial muscle to acetylcholine; nor, in doses up to 10 μg/ml., did it affect responses of guinea-pig or cat bronchial muscle to pilocarpine. This showed both that hexamethonium is virtually devoid of atropinic activity, and also that, when pilocarpine or acetylcholine was used to increase the tone of some preparations before examining the bronchodilator action of nicotine, the rise in tone was not due to nicotine-like ganglionic stimulation.

In addition, hexamethonium has no anti-histamine action. Indeed, in concentrations of 50–100 μg/ml. it augments the histamine response. This might be due to the histamine-potentiating action common to a number of diamines (Arunlakshana *et al.* 1954) or to a subliminal stimulant action by hexamethonium itself. Hexamethonium, up to 400 μg/ml., does not affect the response of guinea-pig trachea to adrenaline or noradrenaline.

From the estimation of anticholinesterase activity of other methonium compounds (Paton & Zaimis, 1949) it might be expected that hexamethonium would have a feeble anticholinesterase action *in vitro*. On the guinea-pig tracheal chain high doses of hexamethonium (up to 500 μg/ml.), can, if added to the bath shortly before, augment the action of a small dose of acetylcholine. But it was found that the response to choline under the same conditions was equally augmented, and that if the hexamethonium was given 15–20 min before acetylcholine or choline the augmentation did not occur, although during the interval a rise of tone might take place. It is therefore likely that the hexamethonium was not exerting any anticholinesterase action, but rather that its own feeble stimulant effect can, under suitable conditions, sum with that of acetylcholine or choline.

Hexamethonium and the anaphylactic bronchoconstriction. Tracheal chains were taken from a guinea-pig sensitized 4 weeks previously by the injection of egg albumin, 100 mg intraperitoneally and a further 100 mg subcutaneously. The responses in the presence of hexamethonium 20 and 200 μg/ml. were compared with those of a control preparation. All three preparations gave a sustained maximal contraction in response to the addition of egg albumin (100 μg/ml.) to the bath, and no difference in the responses due to the hexamethonium could be detected.

DISCUSSION

Using preparations of isolated tracheal and bronchial muscle from guinea-pig and cat, we have demonstrated a number of responses to nicotine, which have the pharmacological characteristics usually attributed to the effects of ganglion-cell stimulation by nicotine. (*a*) The responses were transient, the smooth muscle recovering without removal of nicotine from the bath, and in this state remaining refractory to further doses of nicotine. (*b*) The responses were blocked by very low doses of hexamethonium, by 'nicotinic' alkaloids, and by drugs having a local anaesthetic action. (*c*) The responses were in general blocked by specific antagonists of the appropriate post-ganglionic chemical transmitters. These reactions conform with the known presence of ganglion cells in the respiratory tract.

But the results also point to the presence in the tissue studied of ganglion cells mediating bronchodilator actions in addition to the familiar bronchoconstrictor action. Caution is necessary, however, in assuming that experiments of this type prove the presence of two types of ganglion cell. Nicotine is known to stimulate nerve endings, including sensory nerves in skin, and baroreceptor and chemoreceptor endings (Coon & Rothman, 1940; Brown & Gray, 1948; Ambache & Robertson, 1952; Douglas, 1952; Düner & Pernow, 1952; Diamond, 1955) so that the possibility exists that it may produce its actions by eliciting a local axon reflex from adrenergic or cholinergic nerves, as well as or in place of ganglionic excitation. A crucial experiment to distinguish axonal from ganglion excitation by added drugs is peculiarly difficult to devise. But the known presence of ganglion cells in the bronchi, combined with the fact that hexamethonium in such low concentrations can reduce the effects of nicotine on bronchial muscle, leads us to believe that its actions are, in these experiments, ganglionic. Reports of the use of hexamethonium in antagonizing nicotine on nerve endings usually refer to concentrations of 100 μg/ml. or more, although Diamond (1955) states that with the isolated perfused carotid sinus of the cat the minimum concentration of hexamethonium necessary to block the acceleration by threshold doses of nicotine or acetylcholine of the discharge from the pressure receptors varied from 0·1 to 10·0 μg/ml. Transmission in the superior cervical ganglion is reduced by concentrations of hexamethonium of 1·0 μg/ml. or less. Since the actions of nicotine on the bronchi in our experiments were antagonized by hexamethonium 0·1 μg/ml., we shall assume, in this discussion, that the actions of nicotine are ganglionic; if this proves to be wrong the interpretation we give would require modification.

Macklin (1929), reviewing the somewhat confused literature on the subject, consigns nicotine to a class of drugs which are claimed to be bronchodilator by some, bronchoconstrictor by others, and ineffective by still other authors. In the present work the nature of the bronchial response to nicotine depended on whether or not the preparation had a tone. With guinea-pig trachea, which has considerable tone, or with cat tracheal or bronchial rings maintained in a state of partial contraction by pilocarpine, the primary response to nicotine was dilatation. With preparations which had no resting tone and were hence incapable of dilatation—isolated perfused guinea-pig lungs, cat trachea and bronchi—the primary response to nicotine was bronchoconstriction. On the other hand, the presence of a bronchoconstrictor component to the nicotine response could be demonstrated in all the above preparations by the use of physostigmine. Thus both types of ganglion cell appear to be present in the bronchi of guinea-pigs and cats. The nature of the response to nicotine recorded depends on whether or not the constrictor component is enhanced by eserinization, and, if our deductions about the functions of the ganglion cells in the bronchi are correct, by the relative proportions of intact adrenergic and cholinergic ganglion cells present.

The fact that we were unable to elicit nicotine responses in preparations of guinea-pig secondary bronchi or of the smaller human bronchi does not preclude the presence of ganglion cells or cells of similar type in these preparations. It does suggest the absence of entire cells—soma, axon and ending—in the rings we cut. It is known from anatomical studies that the ganglionic elements are disposed more and more longitudinally as the bronchial tree is descended; ganglion cells are rarely found beyond the proximal ends of third-order bronchi in human lung, though the post-ganglionic fibres continue down to the muscle of the smallest bronchioles (Larsell & Dow, 1933). In cutting rings for the preparations of smaller bronchi the axons of such ganglion cells would be severed.

If there are bronchodilator ganglion cells in the bronchial walls, these may on one hand be aberrant sympathetic ganglia and would be brought into play during a sympathetic discharge. On the other hand it may be that they, together with bronchoconstrictor ganglion cells, receive a preganglionic supply from the vagus nerve; this would provide means whereby vagal stimulation could produce bronchodilatation as well as bronchoconstriction. It is then possible that the vagus maintains the balance of resting bronchomotor tone by playing on both types of ganglion cell. Such alternative routes of bronchial control could offer a physiological advantage to the body. The route through the sympathetic nerves could be called into play as part of a generalized sympatho-adrenal discharge. But if a bronchodilatation independent of general sympathetic activity were required, the vagal pathway with its selective action would be more appropriate.

The nature of the post-ganglionic transmitters

In the experiments on the bronchodilator effect of nicotine on guinea-pig trachea it was demonstrated that the response was antagonized by ergotoxine, ergotamine and dihydroergotamine. Quantitatively, we found that responses to nicotine were antagonized by approximately the same concentration of ergot derivative as that required to antagonize similar responses to adrenaline or to isoprenaline; but noradrenaline effects were antagonized only by considerably larger doses of the alkaloid. This indicates that the post-ganglionic transmitter at the bronchodilator endings in the guinea-pig trachea resembles adrenaline or isoprenaline rather than noradrenaline.

The second response analysed, the transient bronchoconstriction to nicotine, was always abolished by low concentrations of atropine and potentiated by small doses of physostigmine. Transmission at these post-ganglionic endings must be attributed to the muscarinic action of acetylcholine.

The preparations of guinea-pig primary bronchi used showed a more complicated response to nicotine, consisting of three components. The first component was a typical cholinergic bronchoconstrictor response. When this had been abolished by atropine, it was replaced by a transient bronchodilatation comparable to the adrenergic responses seen with trachea. The remainder of the response is not so easily interpreted. It consisted of a long-lasting slowly developing contracture, which resisted atropine, was not potentiated by eserine and was not affected by mepyramine or B_1-pyrimidine in sufficient doses to alter equivalent responses to histamine. This contracture was abolished by hexamethonium or cocaine, and there is therefore reason to believe that it was mediated by ganglion cells. Thus it appears to be a ganglionically mediated slow bronchoconstrictor response, atropine-resistant and not involving histamine. This finding is reminiscent of the observations of Henderson & Roepke (1934, 1935) that there is an atropine-resistant component to the motor action of acetylcholine on the bladder, although these authors, as well as Edge (1955) thought it to be mediated via post-ganglionic parasympathetic neurones. Similarly, Sherif (1935) concluded that the hypogastric nerve in the bitch carried cholinergic fibres to the uterus; the response, though atropine-resistant, was enhanced by eserine. It may be that in the bladder, in the uterus, and in guinea-pig primary bronchi, atropine-resistance is a property of the synapse (as suggested by Dale & Gaddum in 1930) and that the transmission is indeed cholinergic. But with guinea-pig primary bronchi the additional fact that the slow contracture is not potentiated by physostigmine favours a transmitter for this part of the bronchoconstrictor response other than acetylcholine. The growing recognition of the biological role played by slow-reacting substances lends some plausibility to this view.

SUMMARY

1. Nicotine produces a relaxation of the guinea-pig tracheal chain which is abolished by previous treatment with nicotine, hexamethonium, lobeline, coniine, cytisine, sparteine, anagyrine or cocaine.
2. This relaxant action could be antagonized by ergotoxine, ergotamine or dihydroergotamine. The activity of these alkaloids against nicotine was comparable to that against adrenaline or isoprenaline; but much higher doses of the antagonists were needed to block the relaxant action of noradrenaline.
3. Atropine or mepyramine in doses specifically effective against acetylcholine or histamine did not reduce this effect of nicotine.
4. It is suggested that nicotine can excite adrenergic ganglion cells in the tracheal wall, and that these cells release a sympathin resembling adrenaline or isoprenaline rather than noradrenaline.
5. If the tone of a guinea-pig tracheal chain is reduced, or if it is treated with physostigmine, nicotine now produces a bronchoconstriction, abolished by ganglion-blocking agents or by atropine. This is believed to be due to excitation of cholinergic ganglion cells.
6. With cat tracheal rings, which have little normal tone, the first action of nicotine is bronchoconstrictor. If the tone is raised with pilocarpine,

bronchodilator responses to nicotine occur. These reactions have the properties of reactions by cholinergic and adrenergic ganglia respectively.

7. Guinea-pig primary bronchi exhibit a third response to nicotine, a rise in tone of slow onset, long-lasting, resistant to atropine or eserine, unaffected by mepyramine or B_1-pyrimidine, but abolished by hexamethonium or cocaine. This suggests a ganglionic response mediated by some substance other than acetylcholine or histamine, possibly a 'slow-reacting substance'.

8. A number of other preparations of tracheal or bronchial muscle were tested, but offered no advantage over the ring preparations.

9. Hexamethonium was found to be free of atropinic, anti-histamine, or anti-adrenaline action on tracheal ring. In high concentrations, it produced itself some rise of tone not abolished by atropine. Hexamethonium does not modify the anaphylactic response of tracheal muscle.

We should like to express our gratitude to Dr H. G. Herxheimer for suggesting the problem which led to this work.

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STUDIES ON THE DISTRIBUTION OF FACTOR I IN MAMMALIAN BRAIN

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Factor I, whose role as a transmitter substance of inhibitory neurones is under discussion (Florey, 1954, 1956, 1957; Florey & McLennan, 1955*a*, *b*; Edwards & Kuffler, 1957; McLennan, 1957), has been found to occur in the brains of mouse, rat, guinea-pig, rabbit, cat, cattle and horse (Florey, 1954). We may safely assume that it occurs in the brains of all mammals and that conclusions drawn from its behaviour in one type of brain may be applied to other mammalian brains as well. Recent studies have shown that Factor I is produced by brain tissue and that most, if not all, of it is present in an 'occult', inactive form from which it can be released *in vitro* by the action of hypotonic medium, heat, weak alkali or weak acid (Elliott & Florey, 1956). It seems likely that its release *in vivo* is effected by the excitation of certain (inhibitory?) neurones. For crustacean inhibitory neurones this has recently been demonstrated (Florey, 1957).

In mammals Factor I occurs in high concentration only in the central nervous system, but is absent in peripheral nerves, in contrast to crustaceans where Factor I is also found in peripheral nerves (Florey, 1954). These findings have been interpreted as an indication of a functional connexion between Factor I and inhibitory neurones, inasmuch as in mammals inhibitory neurones are found in the central but not in the peripheral nervous system, while in crustaceans they are present in peripheral nerves also. It is obvious, however, that much more detailed information is needed as to the occurrence of Factor I in the various structures of nervous systems in order to ascertain a connexion between location and function of this Factor.

In the present study we attempted to find out whether there is a differential distribution of Factor I within the mammalian central nervous system. We found that the concentrations of Factor I within the different structures and pathways of the brain show wide and consistent variations. We will try to correlate our findings with the available information concerning the inhibitory and facilitatory function of these systems and to relate the distribution of Factor I to the already known data concerning the distribution of acetylcholine, adrenaline, noradrenaline, 5-hydroxytryptamine and substance P.

METHODS

Assays for Factor I were performed on the crayfish stretch receptor preparation according to the method described by Elliott & Florey (1956). The species of crayfish used in all tests was *Cambarus virilis* Hagen. The method permits the determination of the Factor I content of rather small samples; in certain cases we assayed tissue samples as small as 10 mg.

The procedure for the preparation of extracts was as follows: Brains from freshly killed cattle were obtained at local slaughter-houses. Dissection of the brains was usually begun 3 hr after excision. The dissections were completed within 2 hr. Control tests proved that no significant synthesis or loss of Factor I occurred during this dissection period. In one such test we processed the caudate nuclei of several brains, 3, 4, 5 and 6 hr after excision and assayed them in the routine manner. All values in this series were found to be within $\pm 1{\cdot}25\%$ of their average. During the first 2 hr after excision some synthesis may occur (Elliott & Florey, 1956).

Particular care was taken to avoid squeezing or squashing of the tissues, since destruction of cell structures might lead to synthesis or enzymic inactivation of Factor I (Florey, 1954; Elliott & Florey, 1956).

Samples of cortical areas were removed in a cool humid chamber by means of a Stadie–Riggs microtome (Stadie & Riggs, 1944) without moistening. The thickness of the slices was approximately 0·5 mm. Immediately after its dissection each sample was weighed. Larger structures were placed in 9 times their weight of potassium-free, unbuffered saline medium (composition (mм): NaCl 205, $MgCl_2$ 2·6, $CaCl_2$ 13·5). Smaller samples were placed in 49 times their weight of saline medium with half the concentration of potassium which is present in the normal crayfish saline (composition (mм): NaCl 205, KCl 2·7, $MgCl_2$ 2·6, $CaCl_2$ 13·5). All samples were boiled for 5 min in a water-bath and subsequently homogenized. The extracts were kept during the night in the refrigerator at 6° C and assayed on the following day. Dilutions of the extracts were made with the complete saline medium of Elliott & Florey (1956). This solution is made up in the following way: to 1 l. of solution, containing (g) NaCl 12, KCl 0·4, $MgCl_2.6H_2O$ 0·5 and $CaCl_2$ 1·5, are added 100 ml. of 0·11 м tris-(hydroxymethyl) amino methane-maleate buffer, adjusted to give a final pH of 6·5, and 2 ml. of 0·4% bromocresol-purple.

In control tests it was seen that no loss of Factor I occurs during 48 hr of cold storage of extracts. The reproducibility of our assays was found to be within 5%. This was repeatedly ascertained by repetitive assays of one or more samples on one or more different stretch receptor preparations.

The inhibitory activity of the extracts has been determined in crayfish units (c.u.) and compared with a reference solution containing gamma-aminobutyric acid. Crayfish units reference (c.u.r.) have been used as absolute values for the Factor I content of the original tissues. For the derivation of these units see Elliott & Florey (1956). The recent identification of a large part of the Factor I of brain with gamma-aminobutyric acid (Bazemore, Elliott & Florey, 1956, 1957) allows a direct measurement of Factor I activity in terms of the activity of this known substance. We have found in a large number of comparisons that 1 c.u.r. of Factor I can be identified with the activity of 2 μg of gamma-aminobutyric acid. We have used solutions of gamma-aminobutyric acid in all bioassays for Factor I. However, since the total activity of Factor I is not yet completely identified we refrain from giving absolute values for gamma-aminobutyric acid and use the term c.u.r. as a measure of the Factor I content of brain tissue. This seems the more indicated as McLennan (1957) has recently shown that the actions of Factor I and gamma-aminobutyric acid are not always identical.

For the stretch receptors of *Cambarus virilis* it has already been shown (Elliott & Florey, 1956) that none of the neurogenic principles like adrenaline, noradrenaline, 5-hydroxytryptamine, thiamine, acetylcholine, choline, and histamine, of concentrations likely to be present in the extracts assayed, interfere with the action of Factor I. We have prepared substance P according to a method described by Lembeck (1953). Even a solution of this preparation of 1 mg/ml. was without effect on the stretch receptors. It is thus unnecessary to purify the crude extracts or to make use of pharmacological blocking agents for Factor I assays.

The inhibitory action of Factor I on the crayfish stretch receptor preparation is blocked by low concentrations of picrotoxin (Elliott & Florey, 1956). We have repeatedly made use of this fact in order to ascertain that the inhibition caused by the extracts is indeed due to Factor I.

Samples were obtained from a total of sixty-nine beef brains. The animals were not selected for sex or age. Of these, thirty-five were dissected in Montreal and thirty-four in Seattle. No obvious differences were found between the Factor I content of 'eastern' and 'western' beef brains. Such structures as the caudate nuclei which gave rather uniform values for Factor I were particularly suitable for comparisons. The values for six caudate nuclei obtained in Montreal ranged from 202 to 240 c.u.r./g, and the values for five caudate nuclei obtained in Seattle varied from 230 to 255 c.u.r./g.

The revised Nomina Anatomica, as approved by the International Nomenclature Committee, Paris 1955, are used throughout this paper with the exception of the names for cortical areas of bovine brain which have been taken from Papez (1929). Papez gives names of human brain structures which probably correspond to the structures of bovine brain described by him. In Table 2 these corresponding names are given in parentheses. They conform to the above-mentioned Nomina Anatomica. Wherever this is commonly done we have used the anglicized form of the latin names.

RESULTS

As is shown by Tables 1 and 2, there are considerable and consistent differences between the Factor I contents of the various brain structures. The values for the various areas of cerebral and cerebellar cortex are grouped around 100 c.u.r./g with the exception of the parietal cortex, the Factor I content of which was found to be significantly higher (average 177 c.u.r./g).

TABLE 1. Factor I content of bovine brain: White matter

Structure	Activity (c.u.r./g) Individual samples	 Average
Inferior cerebellar peduncle	34, 35, 43	37
Middle cerebellar peduncle	11, 14, 23, 23	18
Superior cerebellar peduncle	86, 101, 105, 111	101
Pyramidal tract	16, 17	16
Pons	32, 54	43
Crus cerebri	116, 116, 160, 160, 166, 181, 210	158
Corpus callosum;		
Genu	36, 42,	30
Body	14,	
Splenium	32	
Fornix	16, 20	18
Optic tract	54, 60, 76, 76, 90, 100	76
Optic nerve	<8, <8, <8, <8	<8
Oculomotor nerve	20	20
Trochlear nerve	30	30
Trigeminal nerve	<12	<12
Dorsal spinal roots	<10, <10, <10	<10
Ventral spinal roots	<10, <10, <10	<10

TABLE 2. Factor I content of bovine brain: Grey matter

Structure	Area	Activity (c.u.r./g)	
		Individual samples	Average
Cerebral cortex			
Motor cortex: Anterior and posterior sygmoid gyri (=inferior and supracentral gyri)	4, 6	110, 142	126
Sensory cortex: Coronal gyrus (=postcentral gyrus)	3	78, 102, 123	110
Postcruciate gyrus (=superior postcentral gyrus)		147	
Anterior ectosylvian gyrus	—	100, 104	
Inferior postcentral gyrus	—	100, 123	
Temporal lobe: Posterior ectosylvian gyrus (=superior temporal gyrus)	41, 42, 22	76, 101, 105	97
Posterior suprasylvian gyrus (=middle temporal gyrus)	21	105	105
Temporal pole	38	75, 85	80
Frontal cortex: Superior and middle frontal gyri	8, 9, 10	63, 86	75
Parietal lobe cortex	—	106, 172, 240, 190	177
Occipital lobe cortex	—	115, 118, 121, 133	122
Insula	—	88, 94	91
Hippocampal gyrus	—	111	111
Cingulate gyrus	—	68, 88, 11	89
Basal ganglia			
Caudate nucleus	—	202, 212, 215, 230, 233, 240, 240, 245, 246, 250, 255, 255	235
Putamen	—	134, 166, 171, 178, 195	169
Globus pallidus	—	205, 333, 350	296
Amygdaloid nucleus	—	107	107
Thalamus			
Whole	—	72, 72, 78	74
Anterior thalamic nucleus	—	80, 74	77
Metathalamus			
Lateral geniculate body	—	106, 127, 150, 182	141
Medial geniculate body	—	85, 133, 153, 162	133
Epithalamus			
Pineal gland	—	10	10
Hypothalamus			
Mamillary body	—	125, 133, 136, 140, 158	138
Infundibulum and tuber cinereum	—	100, 115	107
Hypophysis	—	22	22
Subthalamic nucleus	—	255, 309, 333	299
Mesencephalon			
Inferior colliculi	—	121, 190, 230	180
Superior colliculi	—	230, 277, 300, 307	281
Substantia nigra	—	317, 383, 360, 424, 428, 444, 446, 464	406
Red nucleus	—	60, 77, 117, 182	109
Substantia grisea centralis, random samples	—	100, 105, 187, 225, 240, 240, 380, 454, 454, 477	234
Level of inferior colliculi	—	154, 182	168
Level of posterior border of superior colliculi	—	146, 281	213
Level of anterior border of superior colliculi	—	100, 211	155
Cerebellum			
Cerebellar cortex: Posterior lobe	—	60, 63	62
Anterior lobe	—	65, 116	91
Paraflocular lobe	—	95	95
Anterior lobe: Whole	—	145	145
Dentate nucleus	—	202, 205, 210, 295, 333, 367, 420, 490	315
Rhombencephalon			
Reticular formation:			
Central portion, level of nucleus of XII nerve	—	90, 97	94
Lateral portions, level of nucleus of XII nerve	—	125, 133	129
Central portion, level of exit of VII nerve	—	133, 267	200
Lateral portions, level of exit of VII nerve	—	92	92
Central portion, upper pons	—	183, 177	180
Inferior olive	—	41, 66, 97	68
Dorsal cochlear nucleus	—	133	133
pinal cord			
Posterior horn	—	150	150
Anterior horn	—	75	75

Concentrations similar to those found in most cortical areas are present in the lateral and medial geniculate bodies, the thalamus, the amygdaloid nucleus, inferior olives and the dorsal cochlear nucleus. A number of other grey structures contain conspicuously high amounts of Factor I. In order of increasing Factor I content they are: mamillary bodies (125–158), rhombencephalic reticular formation (90–267), putamen (134–190), inferior colliculi (100–200), mesencephalic substantia grisea centralis (100–477), caudate nuclei (202–255), superior colliculi (230–307), globus pallidus (205–350), subthalamic nucleus (255–333), dentate nuclei (202–490), hypothalamus (375–450) and substantia nigra (316–464 c.u.r./g).

For the grey matter of the spinal cord we found 75 c.u.r./g for the anterior, and 150 c.u.r./g for the posterior horns. Both samples were taken from the cervical region of the cord. Assays of ventral and dorsal spinal roots (lumbar region) indicated activities of less than 10 c.u.r./g (see also Florey, 1954; Florey & McLennan, 1955*a*).

Most of Factor I of the central nervous system thus appears to occur within the grey matter. Only small amounts are found in white matter, cranial nerves, pineal body and hypophysis. This would indicate a close association with the nerve cell bodies or synaptic regions. In three instances, however, conspicuous concentrations of Factor I were found in 'white' fibre tracts. This indicates that this factor is also present within the axons of certain neurones. That these axons must be of a specific functional type is demonstrated by the fact that they are found in and restricted to certain pathways, in contrast to neighbouring tracts which contain almost no Factor I. Of the three cerebellar peduncles only the superior cerebellar peduncle contains relatively large amounts of Factor I, and it is this tract that is known to carry 'inhibitory' fibres from the dentate nucleus (rich in Factor I) to the red nucleus, thalamus, etc. The optic tract is another example of a fibre tract containing larger quantities of Factor I. That the sensory fibres contribute practically nothing to its Factor I content is shown by the fact that the optic nerves contain no detectable quantity of it. We must thus conclude that the optic tract contains non-sensory fibres which are extremely rich in Factor I. It is likely that they represent at least part of the commissural fibres.

The third case of relatively high Factor I content of white matter is that of the crus cerebri. The mass of this structure consists of the cortico-pontine fibres, but it is unlikely that these fibres are responsible for the Factor I content because they connect structures of relatively low Factor I content. We suspect that the Factor I content of the crus cerebri can be ascribed to the pallido-nigral fibres which pass through this structure, connecting the globus pallidus with the substantia nigra, both of which have a high Factor I content. The available anatomical data are unfortunately not detailed enough to rule out the possibility that the area dissected contained cell groups belonging to the neighbouring substantia nigra, which has the highest Factor I content of any brain structure. The pyramidal fibres are unlikely to contribute to the Factor I content of the crus cerebri because the same fibres, dissected from the level of the medulla, contain almost no Factor I.

DISCUSSION

Technical considerations

In spite of a highly accurate assay method there is a wide spread of the values obtained for the Factor I content of a number of brain structures. It is obvious that the variations are particularly great with those structures which are difficult to dissect, like the dentate nuclei. One can assume that the lower values can be attributed to admixed white matter. The variations in the Factor I content of cerebral cortex might, at least in part, be due to the fact that we made slices of uniform thickness but the various cortical areas differ in thickness. If this is so, we must, of course, also assume that cortical areas with seemingly equal Factor I content might in reality contain different amounts of this Factor.

The more compact nuclear masses of the brain, such as caudate nucleus, mamillary bodies and putamen, which are easy to dissect and have well-defined borders, show only 20–30 % variation of Factor I content. In contrast to these uniform values we find 60–80 % variation for dentate nucleus and for mesencephalic substantia grisea centralis. The convolute outline of the dentate nucleus makes it impossible to isolate this structure without contamination by neighbouring white matter. For this reason it seems likely that the higher values are more representative of the Factor I content of this nucleus than the lower ones. A similar reasoning applies to the rhombencephalic reticular formation. Here we are dealing with ill-defined structures consisting of minute cell groups scattered among various fibre tracts. Again it seems reasonable to assume that the higher values are more representative than the lower ones if we are interested in a 'chemical' characterization of the neurones originating in this structure. With the possibility of wide variation, it becomes difficult to judge the difference found between the different areas of the reticular formation. They might represent genuine differences in the Factor I content of these areas, but they might also reflect the irregular distribution of Factor I throughout the reticular formation, a distribution which makes it a matter of chance whether a dissected sample includes more or less of tissue rich in Factor I.

We have tried to analyse the conspicuous variability of the Factor I content of the substantia grisea centralis by assaying samples of successive areas of this structure. Again no correlation between Factor I and area was found and we have to assume that, with regard to neurones containing Factor I, the situation here is similar to that found in the rhombencephalic reticular formation.

Functional considerations

All those centres concerned with the correlation and integration of motor activity contain conspicuously high concentrations of Factor I. Practically all centres that comprise the extra-pyramidal system are known to be involved in excitatory as well as inhibitory actions. If one assumes that there are extra-pyramidal pathways which include synaptic stations in a number of extra-pyramidal nuclei, then all these synapses must be considered excitatory in nature, since they have to activate their particular post-synaptic neurone if the inhibitory impulses are to arrive at their destination. If Factor I is an *inhibitory* transmitter, it should only be released at the end of these inhibitory pathways. If Factor I is at all a transmitter substance, its high concentration within the 'extra-pyramidal centres' could mean one of two things: (*a*) More than one neurone of an inhibitory pathway transmits by releasing Factor I; in this case, Factor I can be inhibitory only at the last synapse but must be excitatory at the preceding synapses. (*b*) Factor I is not involved in transmission by neurones belonging to what may be called polysynaptic inhibitory pathways, but rather it would belong to inhibitory tracts involved in direct inhibition of the activity of one centre by the action of inhibitory fibres originating in another centre; these would be single neurone pathways.

Although excitatory actions of Factor I have been demonstrated by Florey & McLennan (1955*b*) and Florey (1956), we have to await final identification of Factor I until it can be decided whether these actions are indeed due to the same substance that causes inhibition of synaptic transmission (Florey & McLennan, 1955*a*, *b*; Florey, 1956; McLennan, 1957).

It is interesting to compare the distribution of Factor I in the central nervous system with that of acetylcholine, substance P, sympathin (noradrenaline) and 5-hydroxytryptamine. No specific data are available on the distribution of gamma-aminobutyric acid in mammalian brain apart from reports that it does occur largely in the central nervous system, is absent in peripheral nerves, and that grey matter contains more than white matter (Udenfriend, 1950; Roberts, Harman & Frankel, 1951; Tallan, Moore & Stein, 1954). The hypothalamus contains large amounts of all the substances under discussion (MacIntosh, 1941; Zetler & Schlosser, 1953, 1954; Vogt, 1954; Amin, Crawford & Gaddum, 1954). The substantia grisea centralis is another structure which contains large quantities of several 'transmitters', noradrenaline (Vogt, 1954), 5-hydroxytryptamine (Amin, Crawford & Gaddum, 1954), substance P (Zetler & Schlosser, 1953, 1954; Amin *et al.* 1954) and Factor I. There are no data concerning the acetylcholine content of this structure. The substantia nigra contains large amounts of Factor I and substance P (Zetler & Schlosser, 1953, 1954), and judging from the high activity of choline acetylase found in this structure (Feldberg & Vogt, 1948) its acetylcholine content is likely to be high also. Nothing is known, however, about the noradrenaline and 5-hydroxytryptamine content. It can thus be seen that some brain structures contain large amounts of several of these substances. This means that a high content of one 'transmitter' does not exclude the presence of a large amount of another transmitter, and its predominance is not indicated unless it can be shown that the others are indeed present in much smaller quantities.

From the foregoing discussion it must be concluded that, with the data at hand, it is not possible to establish a definite correlation between location and function of Factor I. There is, nevertheless, a characteristic pattern of distribution and our results indicate that there are tremendous differences in the Factor I content of the different brain structures. Since extracts containing Factor I are highly potent in affecting synaptic transmission (Florey & McLennan, 1955*a*, *b*), we assume that the differential distribution of Factor I is actually correlated with a functional differentiation of the brain and that different amounts of Factor I reflect the incidence of a type of neurone which is chemically characterized by its Factor I content. These neurones are definitely not sensory (functionally as well as chemically), they differ also, as shown by their distribution, from cholinergic and adrenergic neurones (for information concerning the distribution of cholinergic and 'adrenergic' tracts, consult the papers by Feldberg & Vogt (1948) and Vogt (1954)). We therefore postulate the existence of a further category of neurones which, because of their Factor I content, we should like to name I-neurones.

SUMMARY

1. With the aid of the crayfish stretch receptor preparation, the Factor I content of a large number of brain structures was determined. Factor I was found to be concentrated within the grey matter of the extra-pyramidal centres. A high content of Factor I was also found in the superior cerebellar peduncle, optic tract and crus cerebri. In general, however, white matter was found to contain only little Factor I.

2. Two alternative explanations for the distribution of Factor I in mammalian brain are offered, both based on the assumption that this factor is a transmitter substance: (*a*) Factor I is contained within neurones concerned with direct inhibition of other nerve centres. (*b*) Factor I is contained within the first and last neurones of polysynaptic extra-pyramidal pathways, in which case it has to fulfil the function of an excitatory transmitter in the first synapses of these pathways.

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THE INFLUENCE OF BACTERIAL EXOTOXINS ON THE ACTIVITY OF THE THYROID GLAND IN DIFFERENT SPECIES

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Harris and his colleagues (Brown-Grant, von Euler, Harris & Reichlin, 1954; Brown-Grant, Harris & Reichlin, 1954*a*, *b*; Harris, 1955) showed that the ^{131}I output method forms a simple and direct technique for observing changes in the activity of the thyroid gland of the rabbit. They found that in this species, under standardized conditions, the rate of release of thyroidal ^{131}I follows an exponential curve and that decreased or increased activity of the gland is associated with a decreased or increased slope of the curve respectively. In rabbits, stress inhibited thyroid activity, the effect being mediated by the hypothalamus and anterior pituitary gland. There was apparently, in this particular species of animal, a reciprocal relationship between the activities of the thyroid gland and the adrenal cortex. Further evidence for such a relationship was provided by a study on the effect of administration of corticotrophin or cortisone on the activity of the thyroid gland of the adrenalectomized rabbit, which showed thyroid inhibition when the concentration in the blood of adrenal steroids was increased. Analogous experiments produced comparable results in the rat (Brown-Grant, 1955, 1956*a*, *b*).

Harris and his colleagues (see Harris & Woods, 1957), on the basis of their work on the adrenalectomized rabbit, have drawn an analogy between these animals and man. They suggest: 'It is likely that the normal human, as well as laboratory animals, responds to emotional or physical stress with an increased activity of the adrenal cortex. It is possible that if, for some reason, the adrenal cortical response fails to occur, and the inhibitory effect of a raised blood concentration of adrenal steroids on TSH secretion is lacking, thyroid hyperactivity may ensue.'

This ingenious attempt to explain the phenomenon of thyroid stimulation in man following stress may be valid. The purpose of the present paper is to draw attention to another possible explanation, namely, that certain species are less susceptible than others to a raised blood concentration of adrenal steroids, and further that in these species stress tends to stimulate rather than to depress thyroid activity. Such a hypothesis is compatible with the facts observed by Harris and his colleagues (Brown-Grant *et al.* 1954; Brown-Grant, *et al.* 1954*a*, *b*).

In this laboratory, during routine post-mortem examination of the thyroid glands of various species treated with large doses of cortisone acetate or 'stressed' with living bacteria or bacterial exotoxins, histological evidence of thyroid inhibition has been a constant finding in the rat, rabbit and mouse but not in the guinea-pig or in the few Rhesus monkeys which have been studied. Indeed, in the latter two species the histological changes suggested increased rather than decreased thyroid activity.

This division of species had previously been noted under certain defined conditions, in their response to cortisone acetate. Both the confusion in the literature regarding the effect of the hormones of the adrenal cortex on immunity, and the discrepancies observed between laboratory and clinical evidence, are resolved once the species difference in response to cortisone is appreciated. Species termed cortisone-sensitive are unable to maintain their body weight under prolonged heavy cortisone administration; γ-globulin synthesis, antibody production and resistance to bacterial infection are depressed or abolished. Species termed cortisone-resistant do not lose weight under heavy cortisone dosage, nor are their γ-globulin synthesis, antibody-forming capacity and resistance to bacterial infection significantly impaired by cortisone. Rats, mice, rabbits and ferrets are all 'cortisone-sensitive', whereas the only animals that have been found to resemble man in his resistance to cortisone are the Rhesus monkey and the guinea-pig (Shewell & Long, 1956; Long, 1957).

In this paper experiments using a sensitive ^{131}I output method are described. These show that bacterial exotoxins when injected into rats, rabbits and mice depress thyroid activity, whereas these toxins stimulate thyroid activity in the Rhesus monkey and the guinea-pig.

METHODS

In order to exclude variations in thyroid function due to the oestrous cycle, male animals were used throughout. The species and approximate weights were: albino mice of the Parkes' strain, 20 g; hooded rats, 180 g; albino guinea-pigs of the Hampstead strain, 450 g; sandy half lop-eared rabbits, 2000 g; Dutch rabbits, 1500 g and Rhesus monkeys, 2500–3000 g.

Mice and rats were fed upon Bruce & Parkes (1949) pelleted Diet 41; guinea-pigs on Bruce & Parkes (1947) pelleted Diet 18 supplemented with 5% skimmed milk, hay and cabbage. The rabbits were fed upon a diet of oats, fishmeal, hay and cabbage. The Rhesus monkeys were given Diet 41 supplemented with cabbage and dates. All animals had tap water to drink with the exception of hypophysectomized rats: these were provided with physiological saline containing 5% glucose and, in addition, with bread and milk. The animals were kept in temperature-controlled rooms between 18 and 20° C.

The bacterial exotoxins

Three preparations of bacterial exotoxin with widely different pharmacological actions were selected.

(1) *Corynebacterium diphtheriae* toxin TP 2776 produced by the Wellcome Research Laboratories, the current laboratory standard diphtheria toxin; Lr/1000 dose = 0·00002 ml.; international Schick unit = 0·00003 ml. (as assayed by the method of Gerwing, Long & Mussett, 1957). The doses for guinea-pigs in a dose-response experiment were (i.u.): Group I, 135; Group II, 45; Group III, 15; Group IV, 5 and Group V, 1·7. The toxin was injected intradermally in 0·2 ml. of sodium chloride solution, 0·9% (w/v), into the depilated flank (two high and two low doses).

In other experiments, to induce marked stress, a standard dose of 30 i.u. was injected intramuscularly. The dose for rabbits was 100 i.u. intramuscularly.

(2) *Shigella shigae* (dysentery toxin), Toxin BI; the current laboratory standard 'shiga' toxin. The doses for guinea-pigs and for rats were their respective LD_{50} doses.

(3) *Clostridium septicum* toxin, V.S. VI; the current laboratory standard toxin. This toxin has the advantage that all the species included in this paper are susceptible to its toxic action. The L^+ dose for a mouse is 2·6 mg. To induce severe stress 50 mg/kg was injected intramuscularly.

Drugs

Thyrotrophic hormone. The International Standard for Thyrotrophin was used (0·074 i.u./mg) (Mussett & Perry, 1955). The Standard was dissolved in saline and injected intraperitoneally in the following doses: mouse, 0·1 mg (0·0074 i.u.); rat, 1·0 mg (0·074 i.u.) and guinea-pig, 2·5 mg (0·185 i.u.).

Iodine. Carrier-free ^{131}I was obtained from the Radiochemical Centre, Amersham. It was diluted in saline and injected intraperitoneally in the following doses: mouse, 2·0 μc/20 g; rat, 5·0 μc/200 g; guinea-pig, 5·0 μc/450 g; rabbit, 5·0 μc/1500 g and Rhesus monkey, 7·5 μc/2500 g.

Propylthiouracil. When specified, finely powdered propylthiouracil was injected intraperitoneally as a suspension in saline, in a dose of 50 mg/kg.

Hypophysectomy

The parapharyngeal approach of Smith (1930) was employed under ether anaesthesia.

Measurement of thyroid activity

The measurement of the rate of release of ^{131}I from the thyroid gland has many advantages over measurement of the rate of uptake. It is not affected by a moderately wide range of plasma iodide levels and therefore of increased renal clearance of iodine due to adrenocortical hyperactivity (see Ingbar, 1953). It probably provides a direct measurement of thyroid hormones released from the gland and therefore a reliable index of the activity of the thyroid gland. This activity can be studied for a long period of time, so that it is possible to distinguish between immediate and delayed effects of toxin, and between the effects of single and repeated stimuli. The iodine-release method also permits the use of 'cross-over' experiments, thus eliminating between-animal variation.

The accuracy of the method can be further increased by treating the animals with propylthiouracil, thus eliminating error due to recirculation of iodine (Wolff, 1951). This drug blocks the secretion of thyroid hormones and by so doing stimulates the anterior pituitary to produce thyrotrophin. Its use will, therefore, increase the sensitivity of experiments designed to show thyroid inhibition and decrease sensitivity of experiments planned to show stimulation of the thyroid gland. Experiments with and without propylthiouracil were carried out.

Animals were injected with ^{131}I 60 hr before the first counts were made in order to allow time for complete absorption of the isotope and thus to provide release curves which maintained a steady logarithmic decrease. Counts for individual animals were calculated as a percentage of the first count, which was taken as 100% of thyroidal iodine at the time of the first count. In each case toxins were administered to the experimental animal groups at this time.

A directional scintillation counter (Ekco type N 599 A) attached to a rate-meter was used. This apparatus is designed for relatively low activity counting of γ-irradiation with a standard (Ekco type N 566) crystal of sodium iodide. A steel collimator, 9·5 mm diam., was fitted so that the area receiving irradiation was reduced to 71 mm². By holding an animal over the head of the collimator tube, so that the gland is directly over the receptive centre of the collimator head, direct counts can be recorded in numbers per second. The vibrations of the needle of the dial of the rate-meter indicate when the gland of the animal is directly over the receptive centre. Thus, by combining small doses of ^{131}I with a 9 mm collimator, receptive centre error due to extrathyroidal ^{131}I is virtually eliminated. Animals were held in position with the exception of hypophysectomized rats (and their unoperated controls) which were placed in a Perspex container (Fig. 1) to avoid handling the operated area. Counts taken on day 1 of each experiment were approximately 20 × background.

Fig. 1. Perspex container for rats.

RESULTS

The effect of hypophysectomy on the rate of release of ^{131}I *from the thyroid glands of rats*

An experiment of Wolff's (1951) was repeated and confirmed; the purpose of this experiment was to prove that the method was capable of demonstrating hypothyroidism due to a deficiency of thyrotrophic hormone (TSH) and a subsequent response to injected thyrotrophin. Two groups of ten hypophysectomized rats, and two groups of ten intact control rats, were used. All animals were treated with propylthiouracil. On the fourth day of the experiment one group of hypophysectomized and one group of control rats were injected intraperitoneally with thyrotrophin. Hypophysectomy decreased the rate of release of ^{131}I. This effect was reversed by thyrotrophin (Fig. 2). The method was, therefore, capable of showing the effect on thyroid activity of varying the amount of thyrotrophin that reached the gland.

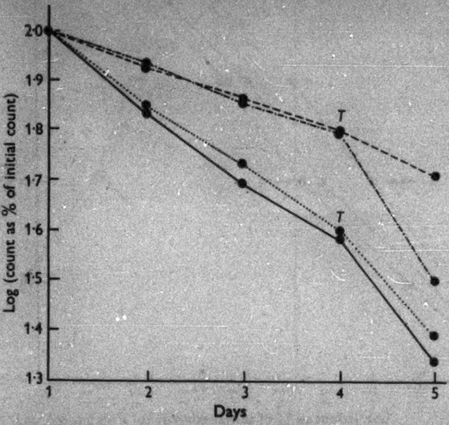

Fig. 2. The effect of hypophysectomy, with and without thyrotrophin, on the rate of release of ^{131}I from the thyroid glands of rats. ——— controls; · · · ·, controls, thyrotrophin at *T*; — — —, hypophysectomized; — · — · —, hypophysectomized, thyrotrophin at *T*.

The effect of bacterial toxins on thyroid activity in various species of animal

The rat. In the first experiment all animals (ten controls and ten treated animals) were treated with propylthiouracil. A statistically significant decrease in the rate of release of ^{131}I from the thyroid glands of intoxicated rats (*Cl. septicum* toxin) occurred at 24 hr ($P = < 0{\cdot}05$), and increased at 48 and 72 hr ($P = < 0{\cdot}001$) (Fig. 3). This was followed by a return to a normal release curve as compared with the control group.

In the second experiment no animals were treated with propylthiouracil, in order to exclude any possible side effect of the drug. The experiment was prolonged and confirmed that the release curve of toxic animals did not differ significantly from that of controls once the toxic phase had passed.

In the third experiment the first experiment was modified by doubling the number of controls (twenty animals) and toxin-treated rats. The previous findings were confirmed. On the third day of the experiment ten controls and ten toxic rats were injected with thyrotrophin. The controls behaved uniformly, suggesting that the thyroid glands of these animals were being maximally stimulated. On the other hand, the rate of release of ^{131}I was stimulated by TSH in the toxic rats (Fig. 4). This finding supports the hypothesis that in rats thyroid inhibition due to stress results from suppression of

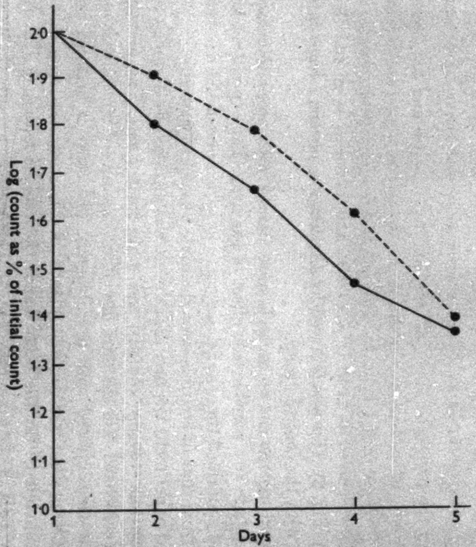

Fig. 3. The effect of *Cl. septicum* toxin on the rate of release of ^{131}I from the thyroid glands of propylthiouracil-treated rats. ———, controls; — — —, stressed.

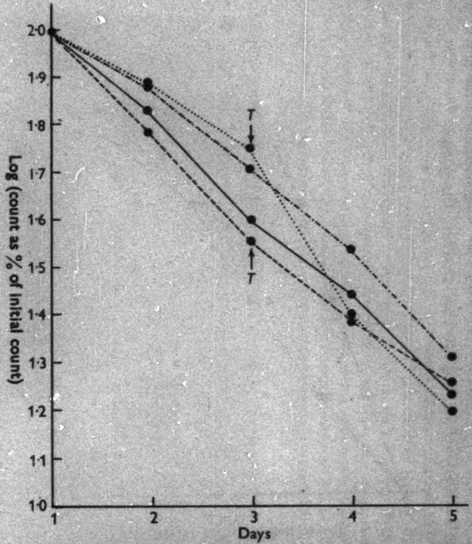

Fig. 4. The effect of thyrotrophin on the rate of release of ^{131}I from the thyroid glands of toxic and non-toxic rats. ———, controls; — — —, controls, thyrotrophin at *T*; — · — · —, stress; · · · · ·, stress, thyrotrophin at *T*.

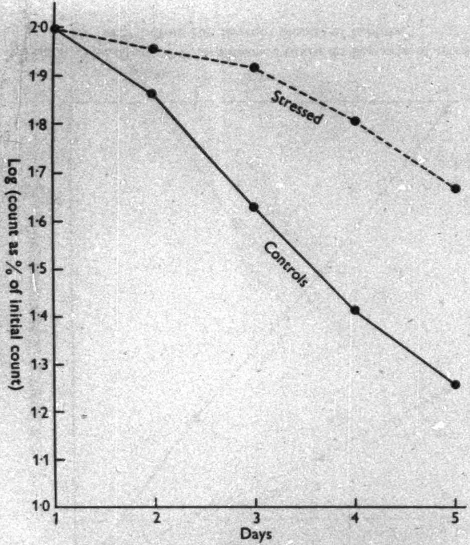

Fig. 5. The effect of an LD_{50} dose of *Sh. shigae* toxin.

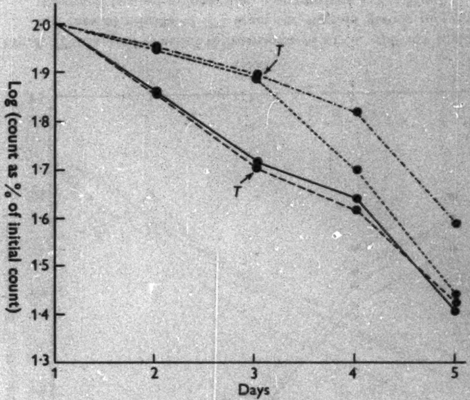

Fig. 6. The effect of thyrotrophin on the rate of release of ^{131}I from the thyroid glands of toxic (*Cl. septicum* toxin) and non-toxic mice. ———, controls; — — —, controls, thyrotrophin at *T*; —·—·—, stress; - - - - -, stress, thyrotrophin at *T*.

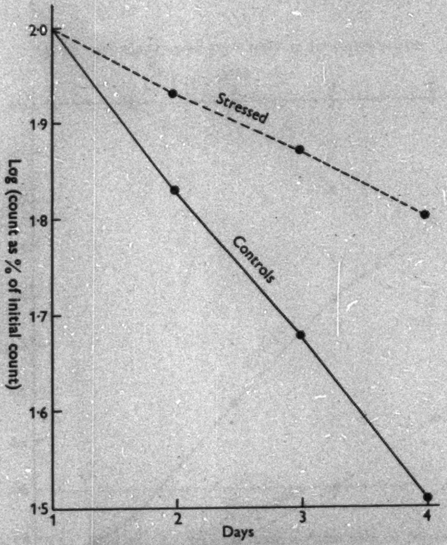

Fig. 7. The effect of *C. diphtheriae* toxin on the rate of release of ^{131}I from the thyroid glands of rabbits.

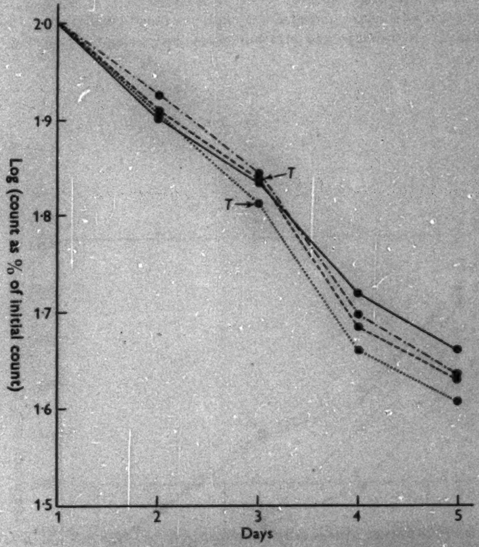

Fig. 8. The lack of effect of thyrotrophin or of *Cl. septicum* toxin on the rate of release of ^{131}I from the thyroid glands of propylthiouracil-treated guinea-pigs. Conventions as for Fig. 4.

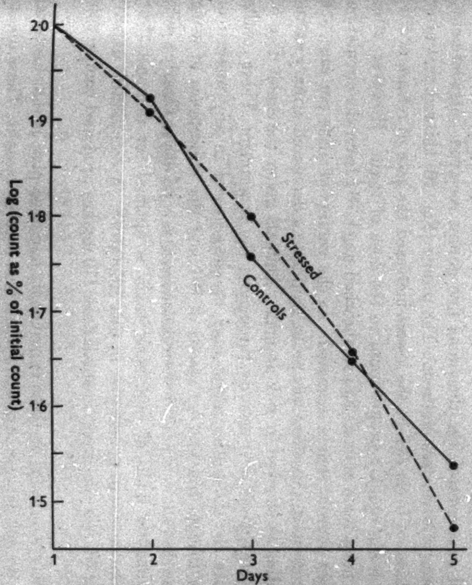

Fig. 9. The lack of effect of *Sh. shigae* toxin (LD_{50}) on the rate of release of ^{131}I from the thyroid glands of propylthiouracil-treated guinea-pigs.

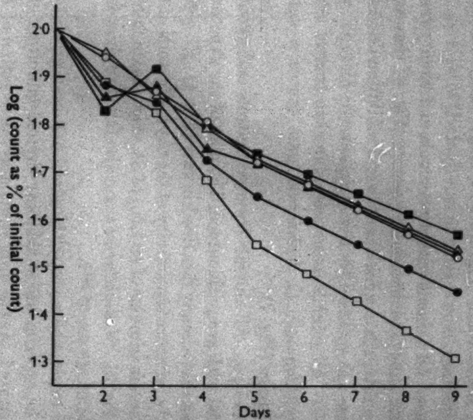

Fig. 10. The effect of graded doses of *C. diphtheriae* toxin on the rate of release of ^{131}I from the thyroid glands of guinea-pigs. ○, control; Schick test doses: △, 1·7; □, 5; ●, 15; ▲, 45; ■, 135.

secretion of TSH. Comparable significant effects were obtained with an LD_{50} of *Shigella shigae* toxin (Fig. 5). This large dose of toxin produced marked thyroid inhibition.

The mouse. All animals (twenty controls and twenty treated animals) were treated with propylthiouracil. The results (Fig. 6) were comparable to those obtained in rats; again significant thyroid inhibition due to toxic (*Cl. septicum* toxin) stress was reversed by thyrotrophin.

The rabbit. All animals (ten controls and ten treated animals) were treated with propylthiouracil. *C. diphtheriae* toxin caused a significant decrease in thyroid activity (Fig. 7).

The guinea-pig. All animals (twenty controls and twenty treated animals) were treated with propylthiouracil. The plan of the experiment was essentially the same as that already described in rats (Fig. 4). *Cl. septicum* toxin did not significantly influence the activity of the thyroid glands of this species (Fig. 8). The more severe stimulus of an LD_{50} of *Sh. shigae* toxin failed to inhibit thyroid function (Fig. 9). *Cl. diphtheriae* toxin also failed to show any significant effect on the rate of release of ^{131}I.

In these experiments propylthiouracil had been given to increase the sensitivity of the experiments so that any thyroid-inhibiting effect of toxin might be demonstrated. As the drug stimulates the secretion of TSH by the anterior pituitary gland, the demonstration of thyroid stimulation in such experiments is virtually impossible. Experiments were therefore carried out in which propylthiouracil was discarded. Six groups of eight guinea-pigs were used; one served as a control, the remaining five groups received, intradermally, graded doses of toxin (see Methods). The results (Fig. 10) showed that after 48 hr all doses of toxin except the lowest increased the rate of release of ^{131}I, the effect being roughly proportional to the dose of toxin. However, after 24 hr, the most toxic animals had re-absorbed much ^{131}I from the circulation so that their thyroid counts increased. As the volume of urine per cage was greatly diminished, this effect was probably due to a decreased urinary excretion of iodine in shocked or sick animals. Animals given the small dose of 5 i.u. showed thyroid over-activity throughout the experiment. These results demonstrate the difficulties which may be encountered in any method of estimating thyroid function by means of ^{131}I and the importance of using a technique that allows repeated observations over several days. Clearly a dose of approximately 5 i.u. of diphtheria toxin is optimal for demonstrating thyroid stimulation in guinea-pigs in this laboratory. A comparable experiment using *Cl. septicum* toxin in small doses (10 mg/kg) produced comparable effects.

The conclusion, based on these experiments in guinea-pigs, is that bacterial exotoxins do not depress thyroid function but may increase it. These observations are the more remarkable because this species has small thyroxine requirements and a low basal metabolic rate, and increased thyroid activity is not readily induced.

Rhesus monkeys. In view of the high cost of Rhesus monkeys and histological evidence that the thyroid glands of monkeys were likely to be stimulated, and certainly not depressed, by toxin, propylthiouracil was not given to these animals. *Cl. septicum* toxin (50 mg/kg) was injected into the adductor muscles of the left hind leg. Thyroid stimulation resulted (Fig. 11). Five controls and five stressed animals were used in this experiment.

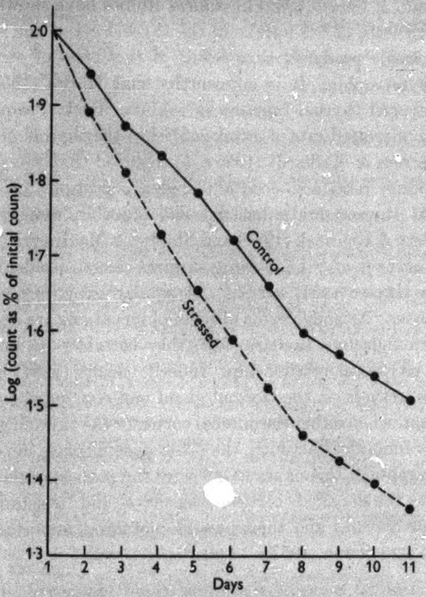

Fig. 11. The effect of *Cl. septicum* toxin on the rate of release of ^{131}I from the thyroid glands of Rhesus monkeys.

TABLE 1. The effect of *Clostridium septicum* toxin on the rate of release of ^{131}I from the thyroid glands of Rhesus monkeys

Time (days)	^{131}I retention (%)		Degrees of freedom	*t*	*P*
	Control	Stressed			
2	75·5±3·7	65·2±7·1	8	3·13	0·01–0·02
3	68·8±3·1	54·3±6·3	8	5·63	<0·001
4	87·4±2·1	77·8±5·3	8	8·02	<0·001
5	60·9±2·9	46·1±5·5	4	9·77	<0·001
8	40·6±2·8	28·4±6·8	4	5·83	0·001–0·01
11	32·4±4·4	24·2±5·7	8	5·91	0·001

DISCUSSION

The facts described in this paper confirm those of other workers. Thus various stress agents inhibit thyroid function in the rat and rabbit as shown by Harris and his colleagues (Brown-Grant *et al.* 1954*a*). The mouse behaves in like fashion. There is, however, a clear-cut species difference in the response of the thyroid gland to stress. The guinea-pig and Rhesus monkey show stimulation of thyroid activity. In this respect they probably resemble man.

Reiss, Forsham & Thorn (1949) in clinical studies have shown that adrenaline increased the rate of ^{131}I uptake of the thyroid gland and suggested that the adrenal steroids produced as a result of this type of stress facilitated secretion of thyrotrophin. It is noteworthy that Harris (1955) found that adrenaline depressed thyroid function in rabbits. Beck, Pasquier & Béraud (1956) found an increased rate of uptake of ^{131}I in the thyroid glands of febrile patients. Engstrom & Markardt (1955*a*, *b*) reported that the serum levels of precipitable iodine remained constant under conditions of severe stress, suggesting that thyroid inhibition did not occur in man under stressful conditions. Perry & Gemmell (1949) and Shipley & MacIntyre (1954) describe an increased turnover of ^{131}I following surgical stress. Goldenberg, Lutwak, Rosenbaum & Hayes (1954) carried out similar experiments and reached similar conclusions. Although some of these observations are open to criticism on technical grounds (see Ingbar, 1953) they certainly do not support the hypothesis of reciprocal relationship. Indeed, Means (1954) considered the evidence in his lectures on the thyroid gland and concluded 'one is tempted to generalise that, when either the adrenal cortex or the thyroid gland increases or decreases its functional activity, the other does likewise in compensation'. It is not the purpose of this paper to consider the more speculative aspects of the division into the so-called 'cortisone-sensitive' and 'resistant' species: the other analogous features and their possible potential importance have been discussed elsewhere (Long, 1957).

SUMMARY

The effect of bacterial exotoxins on thyroid function of various animal species has been studied. It was shown that:

1. A single injection of toxin depressed the rate of release of ^{131}I from the thyroid gland of the rat, mouse and rabbit.
2. A single injection of toxin increased the rate of release of ^{131}I from the thyroid gland of the guinea-pig and Rhesus monkey.
3. A single injection of thyrotrophin (TSH) stimulated thyroid function in rats, mice and rabbits, the activity of whose glands had been depressed by toxin.

4. The hypothesis of a reciprocal relationship between the secretion by the anterior pituitary gland of corticotrophin and thyrotrophin is compatible with the evidence obtained in the rabbit, rat and mouse, but not with that obtained in the Rhesus monkey and the guinea-pig.

5. It is suggested that the response of man to stress is analogous to that of the guinea-pig and Rhesus monkey.

6. It is suggested that in guinea-pig and Rhesus monkey, and possibly in man, the inhibitory effect on thyroid activity of a raised blood concentration of adrenal steroids does not occur. This is attributed to an inherent resistance of these species to the thyroid inhibiting effect of adrenal steroids.

The authors wish to thank Dr D. M. V. Parrott for performing hypophysectomies on the operated rats used in this work.

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THE EFFECT OF CONTINUED TOXIC STRESS ON THE ACTIVITY OF THE THYROID GLAND IN THE RAT AND THE GUINEA-PIG

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It has been shown that a single injection of a bacterial exotoxin has a varying effect on the thyroid function of different animal species. In the rat, rabbit and mouse such a stimulus causes inhibition of thyroid activity, whereas toxic stress causes thyroid stimulation in the guinea-pig and Rhesus monkey (Gerwing, Long & Pitt-Rivers, 1958). In this paper further experiments are described which were carried out to study the effect of repeated small doses of toxin on thyroid activity. The rat and the guinea-pig were used exclusively in this work, the rat representing those species whose thyroid glands were inhibited by toxic stress and the guinea-pig those whose thyroid glands were stimulated.

METHODS

In order to exclude variations in thyroid function due to the oestrous cycle, male animals were used throughout. The species were hooded rats with approximate weights of 180 g and albino guinea-pigs of the Hampstead strain of 450 g. The rats were fed on Bruce & Parkes's (1949) pelleted Diet 41 and the guinea-pigs on Bruce & Parkes's (1947) pelleted Diet 18 supplemented with hay, cabbage and 5% skimmed milk. The animals drank tap water *ad lib.* and were maintained in temperature-controlled rooms between 18 and 20° C.

Bacterial exotoxins

Corynebacterium diphtheriae toxin, TP 2776 produced by Wellcome Research Laboratories; the current laboratory standard diphtheria toxin (Lr/1000 = 0·00002 ml.; international Schick unit = 0·00003 ml.). The dose used in guinea-pigs to induce low-grade continuous stress was 3 i.u. and was injected intramuscularly.

Clostridium septicum toxin V.S. VI, the current laboratory standard toxin; the L^+ dose for a mouse is 2·6 mg. To induce low-grade continuous stress in both guinea-pigs and rats a dose of 10 mg/kg body weight was injected intramuscularly.

Iodine

Carrier-free ^{131}I was obtained from the Radiochemical Centre, Amersham. It was diluted in sodium chloride solution, 0·9% (w/v), and injected intraperitoneally. Both rats and guinea-pigs were injected with tracer doses of 5 μc.

Measurement of thyroid activity

The procedure has been described in the preceding paper (Gerwing *et al.* 1958).

RESULTS

The effect of continued toxic stress on thyroid function in the rat

Two groups of ten male rats were used; one group received injections of *Cl. septicum* toxin twice weekly, and the other group acted as normal controls. Counts were recorded throughout the experiment which was continued for 24 days (Figs. 1, 2). Toxin injections significantly decreased the rate of release of ^{131}I from the thyroid glands of stressed rats. However, 10 days after the experiment there were apparently normal rates of release of ^{131}I from the thyroid glands of the stressed animals; this was followed finally by a phase of what appeared to be thyroid stimulation, in which the rate of release of ^{131}I from the thyroid glands of the stressed rats was significantly greater than that of the controls.

TABLE 1. The effect of continuous low-grade stress with *Clostridium septicum* toxin on the rate of release of ^{131}I from the thyroid glands of rats

Time (days)	^{131}I retention (%)		Degrees of freedom	*t*	*P*
	Control	Stressed			
2	82·7±3·9	91·0±3·9	18	2·89	0·01
3	59·4±3·8	71·8±4·6	18	3·69	0·01–0·001
4	49·6±2·4	52·3±5·5	18	1·00	>0·1
5	39·6±3·9	47·0±3·7	18	3·97	<0·001
8	21·6±3·2	22·8±3·7	18	0·55	>0·1
9	14·5±2·6	19·0±3·9	18	2·11	0·05
10	13·1±1·4	13·5±1·7	18	0·00	>0·1
16	81·4±3·8	80·7±3·6	18	0·15	>0·1
17	63·8±3·1	66·7±3·3	18	1·75	0·05–0·1
18	51·8±4·2	53·6±3·6	18	0·74	>0·1
19	43·4±2·4	41·1±2·7	18	0·73	>0·1
22	25·1±1·8	23·2±2·7	18	1·19	>0·1
23	20·5±1·0	16·8±1·9	18	10·38	<0·001
24	16·3±1·9	11·1±1·5	18	5·29	<0·001

The effect of continued toxic stress on thyroid function in the guinea-pig

Two experiments were carried out on guinea-pigs (ten controls and ten treated animals) similar to that mentioned above for the rat. The first involved the use of diphtheria toxin and was continued for 12 days (Fig. 3). It was seen that the thyroid glands of these animals were apparently unaffected by this toxin for the first 5 days of the experiment, after which the rate of release of ^{131}I from the thyroid glands of the stressed animals increased continuously until the 12th day, when the rate of release was significantly greater than that of the controls.

The second experiment on guinea-pigs involved the injection of *Cl. septicum* toxin and was continued for 24 days (Figs. 4, 5). Here, as in the previous experiment, the rate of release of ^{131}I from the thyroid glands of the stressed animals was unaffected for the first 5 days of the experiment. From that time

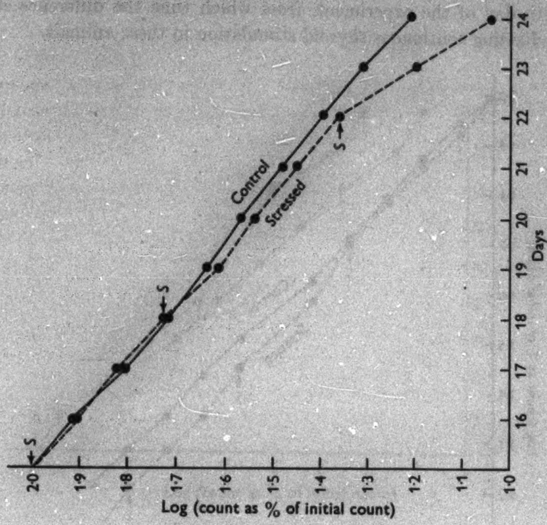

Fig. 2

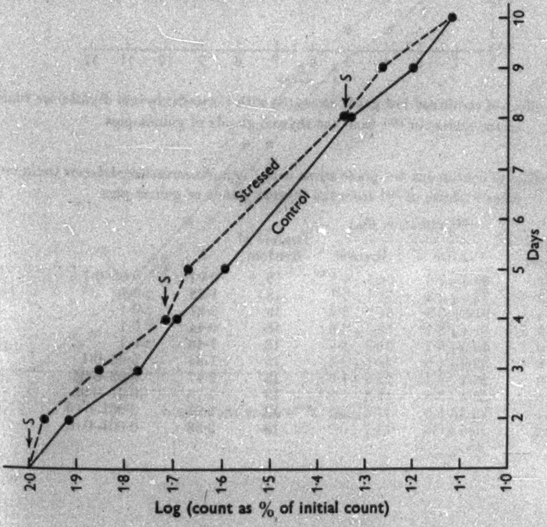

Fig. 1

Figs. 1, 2. The effect of continuous low-grade stress (*S*) with *Clostridium septicum* toxin on the rate of release of ^{131}I from the thyroid glands of rats.

the difference between the two groups increased but did not become significant until the 18th day of the experiment, from which time the difference still increased, indicating continuous thyroid stimulation in these animals.

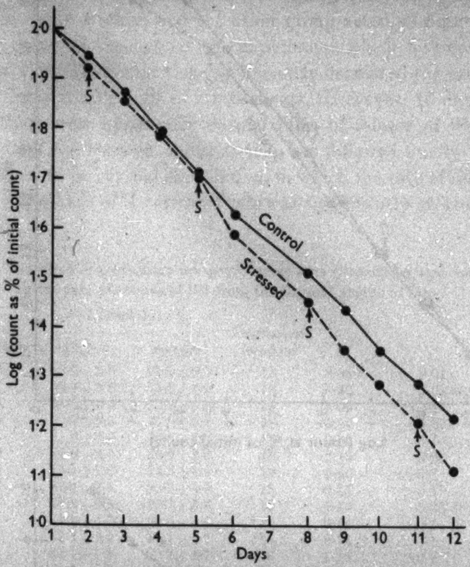

Fig. 3. The effect of continued low-grade stress (*S*) with *Corynebacterium diphtheriae* toxin on the release of ^{131}I from the thyroid glands of guinea-pigs.

TABLE 2. The effect of continuous low-grade stress with *Corynebacterium diphtheriae* toxin on the rate of release of ^{131}I from the thyroid glands of guinea-pigs

Time (days)	^{131}I retention (%)		Degrees of freedom	*t*	*P*
	Control	Stressed			
2	88·6±3·0	84·5±4·1	18	2·01	0·05–0·1
3	75·0±4·3	72·1±3·7	18	1·28	>0·1
4	61·9±3·8	61·5±5·4	18	0·21	>0·1
5	51·4±3·7	50·5±3·6	18	0·44	>0·1
6	43·0±5·1	38·9±3·1	18	1·49	>0·1
8	32·9±4·6	28·5±2·4	18	1·84	0·05–0·1
9	28·1±4·4	22·8±1·6	18	2·47	0·02–0·05
10	23·3±2·8	19·3±1·8	18	2·76	0·01–0·02
11	19·5±0·9	17·4±0·7	18	2·87	0·001–0·01
12	16·5±0·6	14·7±0·7	18	2·99	0·001–0·01

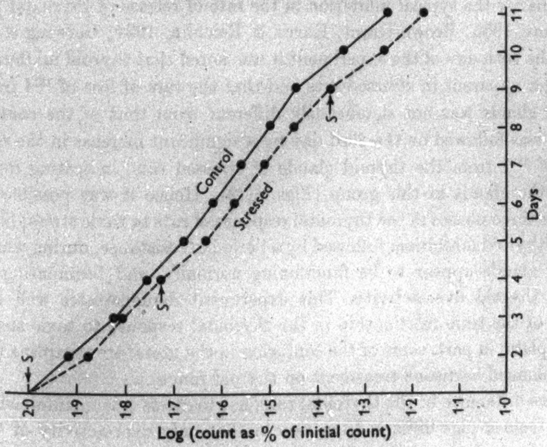

Fig. 4

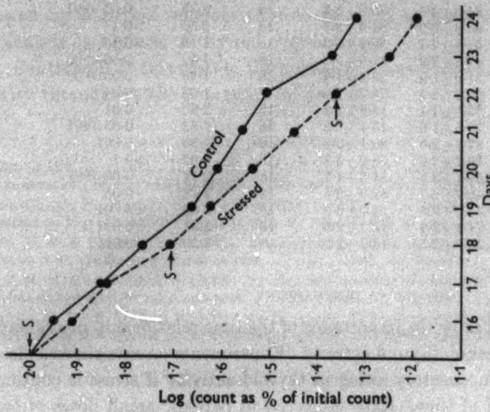

Fig. 5

Figs. 4, 5. The effect of continued low-grade stress (*S*) with *Clostridium septicum* toxin on the rate of release of ^{131}I from the thyroid glands of guinea-pigs.

TABLE 3. The effect of continuous low-grade stress with *Clostridium septicum* toxin on the rate of release of ^{131}I from the thyroid glands of guinea-pigs

Time (days)	^{131}I retention (%)		Degrees of freedom	*t*	*P*
	Control	Stressed			
2	84·0±3·6	75·0±5·9	18	2·33	0·05–0·01
3	66·8±3·6	64·6±2·1	18	0·50	>0·1
4	57·8±2·1	53·1±3·3	18	1·13	>0·1
5	47·9±3·9	42·6±2·2	18	1·41	0·05–0·02
8	31·6±1·9	28·2±1·4	18	1·45	0·05–0·02
9	28·2±2·4	23·7±2·6	18	1·98	0·05
10	22·5±1·7	18·8±1·9	18	2·86	0·01
11	18·6±1·6	15·1±1·3	18	2·52	0·05–0·01
16	87·7±2·5	80·1±3·2	18	4·86	<0·001
17	70·2±2·9	68·9±3·8	18	0·63	>0·1
18	57·1±2·1	51·5±4·7	18	2·14	0·05–0·01
19	46·7±2·7	42·9±2·5	18	1·96	0·05
22	32·0±3·5	24·0±3·9	18	3·63	<0·001
23	24·3±2·4	18·3±2·5	18	3·31	<0·001
24	20·7±2·5	15·9±2·5	18	3·59	<0·001

DISCUSSION

These experiments show the importance of the time relationship in evaluation of the effect of stress on thyroid activity. Experiments on rats showed clearly that there is a compensatory swing in thyroid activity if stress is continued over a long period of time. Rats subjected to periodic small doses of toxin showed primarily the typical inhibition in the rate of release of thyroidal ^{131}I (Brown-Grant, 1956; Brown-Grant, Harris & Reichlin, 1954; Gerwing *et al.* 1958). On the 15th day of the experiment it was noted that thyroid inhibition was no longer apparent in stressed rats and that the rate of loss of ^{131}I from the thyroid glands was not significantly different from that of the control group. This was followed on the 23rd day by a significant increase in the rate of release of ^{131}I from the thyroid glands of stressed rats, indicating over-activity of the glands in this group (Figs. 1, 2). Hence it was possible to demonstrate three phases in the thyroidal response of rats to toxic stress; first, the stage of thyroid inhibition, followed by a period of resistance, during which the thyroid glands appear to be functioning normally, and terminating in a phase of thyroid over-activity. This experiment demonstrates well the importance of the time relationship in the thyroidal response to toxic stress and may explain, in part, some of the confusion in the literature regarding the effect of prolonged cortisone treatment on thyroid function.

The species difference in the thyroidal response to stress was maintained in this work. Guinea-pigs differed from rats in that only over-activity of the thyroid gland was demonstrable throughout the experiments.

SUMMARY

The effect of continuous low-grade toxic stress on thyroid function of rats and guinea-pigs has been studied. It was shown that:

1. Such stimulus in rats causes primarily a depression in thyroid function followed by a period wherein thyroid function appears to be normal and terminating in a phase of thyroid over-activity.

2. Continuous toxic stress in the guinea-pig causes increasing thyroid over-activity throughout the experimental period.

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THE METABOLISM OF SOME THYROID HORMONES BY LIMB-BONE RUDIMENTS CULTIVATED *IN VITRO*

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In previous experiments it was shown that thyroxine (T_4) (Fell & Mellanby, 1955) and triiodothyronine (T_3) (Fell & Mellanby, 1956) added to the culture medium affected the growth and development *in vitro* of the cartilaginous limb-bone rudiments of 6-day chick embryos. Qualitatively the two hormones had similar effects. Both accelerated the histological development of the five rudiments investigated, the femur, tibia, humerus, ulna and radius; they also produced a constant differential effect on the growth in length of the various rudiments, whereby growth was inhibited in different degrees in the tibia, femur, humerus and ulna in that order, but stimulated in the radius. Quantitatively, T_3 was roughly four times as potent as T_4 in retarding the growth of the femur and tibia.

These results were thought to indicate that T_4 and T_3 had a direct action on the explants. Gross & Pitt-Rivers (1953), however, suggested that triiodothyronine might be the active form of the thyroid hormone at the peripheral level, resulting from a partial de-iodination of thyroxine. It was thought that in a simple system such as that provided by organ cultures *in vitro* it would probably be relatively easy to detect de-iodination or other chemical transformations taking place in the iodothyronine molecules. It was decided therefore to grow embryonic bones in the presence of ^{131}I-labelled thyroxine and triiodothyronine and examine both the bones and culture medium after incubation to see whether any transformation had occurred in the iodothyronine molecule. T_3 and the iodothyroacetic acids were thought to be likely metabolites of T_4.

Two main groups of experiments were made. In the first the growth of the bone rudiments in medium to which triiodothyroacetic acid (TA_3) or tetraiodothyroacetic acid (TA_4) had been added, was studied, and the effect of TA_3 on the explants was directly compared with that of T_3. Control experiments were also made with potassium iodide. In the second group, radioactive T_4 or T_3 was introduced into the medium, and subsequently the break-down products of the hormones present in the rudiments, in the medium on which the bones were grown and in 'blank' medium incubated without explants, were identified by chromatographic analysis.

METHODS

Material. The femora, tibiae, humeri, ulnae and radii of 6–8-day chick embryos were cultivated. The 6-day rudiments were entirely cartilaginous, but the degree of development varied somewhat in different chicks; thus in some embryos all the rudiments consisted of early, small-celled cartilage, whereas in others cellular hypertrophy had already begun in the shafts of the femur and humerus, and sometimes also in the tibia, ulna and radius. In the 7-day embryos hypertrophy and early periosteal ossification were usually present in all the five rudiments studied and in the 8-day bones the hypertrophic cartilage was at an advanced stage of differentiation.

Experiments of group 1

Culture technique. The explants, which were all 6-day rudiments, were grown in watch glasses 4 cm in diameter, enclosed in a moist chamber, by the technique previously described (Fell & Robison, 1929; Fell & Mellanby, 1955). The culture medium consisted of three parts of cock plasma and one part of embryo extract; the extract was made by thoroughly mincing and grinding a 13-day embryo and mixing the minced tissue with an equal volume of Tyrode solution supplemented with 1% glucose. In addition, a more dilute extract was prepared by mixing one part of embryo mince with two parts of Tyrode solution not supplemented with extra glucose.

The substances to be tested were introduced into the medium as follows. TA_3, T_3 and KI were weighed into a sterile tube and sterilized with a few drops of absolute ethanol, which was then evaporated off; the compounds were dissolved in Tyrode solution and added to the plasma immediately before use to give a concentration of approximately 16 μg/100 ml. of the final culture medium, the same quantity of Tyrode solution alone being added to the control medium. TA_4 was dissolved in ethanol and added to the plasma so that the final medium contained 16 μg TA_4/100 ml. and 0·2% ethanol; the same amount of ethanol was introduced into the controls.

The newly dissected rudiments were washed several times in Tyrode solution, and the dilute embryo extract was then substituted for the saline. The explants were transferred to the clot with a pipette and the surplus extract was removed with a very fine pipette; one of each pair of rudiments was placed on medium containing the agent to be tested and the other on control medium. Every 2 days the rudiments were removed from the old clot, washed in Tyrode solution, followed by dilute embryo extract, and transferred to fresh medium; they were grown for 8 days.

Measurement of growth in length. The bones were drawn with the aid of a camera lucida when first explanted and thereafter at 2-day intervals; their lengths were measured in the manner previously described (Fell & Mellanby, 1955).

Two experiments were made with each of the three compounds mentioned above. Three embryos were used for every experiment, so that each compound was tested on six paired femora, tibiae, humeri, ulnae and radii. The average growth rate of the six experimental and control explants was calculated for each rudiment and each compound.

Histological methods. The explants from one experiment with TA_3 and one with TA_3 and T_3 were fixed for 20–30 min in 3% acetic Zenker's fluid followed by 45 min in Zenker's fluid without acetic acid; they were embedded in paraffin wax and serially sectioned. The sections were stained with Delafield's haematoxylin and chromotrop.

Experiments of group 2

Culture technique. The rudiments were explanted in large watch-glasses 6 cm in diameter enclosed in 10 × 1·5 cm Petri dishes; the dishes were carpeted with cotton-wool saturated with 20 ml. of sterile distilled water. Each watch-glass contained 1·5 ml. plasma and 0·5 ml. embryo extract. In some experiments (see Table 1) the concentrated embryo extract described above was used, but in others the extract was much more dilute (1 part embryo mince: 4 parts Tyrode solution supplemented with 1% glucose).

Synthetic T_4 and T_3 labelled in the 3′, 5′ or 3′ positions were used. Biosynthetically labelled hormones were also prepared by alkaline hydrolysis of the thyroid glands of mice after injection of ^{131}I and chromatographic separation. The labelled T_4 and T_3 were dissolved in a few drops of 0·1 N-NaOH and were then taken up in carrier solutions of T_4 and T_3. The solutions were added to the plasma shortly before use, as described above, to give concentrations of approximately 16 μg/100 ml. of medium.

In four experiments (see Table 1) 6–8 femora and tibiae from 6-, 7- or 8-day chick embryos were explanted in each watch-glass on medium containing labelled T_4 or T_3. In one experiment (no. 183) the bones were grown for 6 days in the presence of unlabelled T_4 by the method used for group 1, and were then transferred to large watch-glasses containing T_4, six bones being placed in each vessel; in another experiment (no. 204) the rudiments were grown for 4 days in the presence of unlabelled T_3 before being transplanted to medium containing the labelled T_3. A 'blank' clot containing the labelled hormone but without explants was prepared in each experiment.

After 18–22 hr growth in labelled medium the cultures were harvested aseptically. With a medium-sized pipette the explants with the fluid immediately surrounding them were collected in one tube, the plasma: embryo-extract clot on which they had grown was placed in another and the 'blank' clot in a third. The material was analysed within 24 hr of harvesting.

Analysis of radioactive products in (a) bones and (b) medium. After incubation the bones were separated from the medium, finely chopped in 0·2 ml. methanol-ammonia (3:1) and mechanically shaken for 15 min. The resulting gelatinous solution, the medium separated from the bones, the control medium and a sample of the original ^{131}I-labelled hormone were analysed by ascending chromatography in butanol:dioxan:2N-NH_3 (4:1:5). Thyroxine, triiodothyronine and iodide were added as markers. The radioactivity on the chromatograms was detected by scanning with a recording strip counter and quantitative results were obtained from the record by measuring the area under each peak. Iodide was visualized by staining with 0·1% palladium chloride; the iodothyronines were detected with diazotized sulphanilic acid as previously described (Gross & Leblond, 1951).

RESULTS

The behaviour of 6-day rudiments grown in the presence of TA_3, TA_4 and KI

The effect of TA_3. In each of two experiments, the femora, tibiae, humeri, ulnae and radii were removed from three 6-day embryos; one of each pair of bones was cultivated for 8 days on medium containing 16 μg/100 ml. medium and the other on control medium.

During the first 2 days in culture there was little or no difference between the experimental and control explants, but by the 4th day the ends of all the TA_3-treated rudiments were larger, and in the femora, tibiae and humeri (but not in the ulnae and radii) the shafts were shorter than in the corresponding controls. These differences became more conspicuous during the next 2 days, when the TA_3-treated bones appeared thick and clumsy as compared with their more slender and shapely controls.

The average growth rates of the rudiments were estimated from the serial camera lucida drawings mentioned in Methods. The results showed that TA_3 produced a similar type of differential effect (Fig. 1*A*) on the growth in length of the various rudiments to that previously observed with T_4 and T_3 (Fell & Mellanby, 1955, 1956).

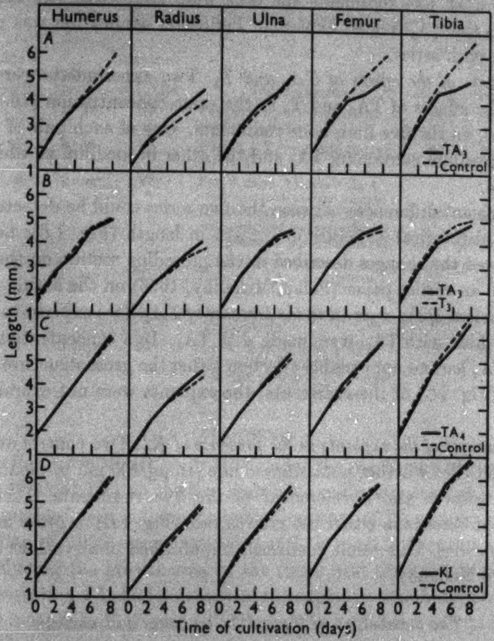

Fig. 1. Graphs showing the average growth in length of paired limb-bone rudiments during cultivation in normal medium and when exposed to various chemical compounds. Each graph represents six pairs of rudiments; the concentration of all the compounds tested was approximately 16 μg/ 100 ml. of medium. *A*, TA_3; the growth of the tibia, femur and humerus has been diminished in that order of degree, there is no significant effect on the ulna and the growth of the radius has been increased. *B*, TA_3 and T_3; the effects of the two agents are similar. *C*, TA_4; little or no effect. *D*, KI; no effect.

A comparison of the histological structure of the TA_3-treated and control explants showed that TA_3 produced the same type of effect as that already described for T_4 and T_3. In all five rudiments the hypertrophy of the cartilage cells had spread precociously into the proliferative zones of flattened cells; in the three wing-bones these zones were greatly reduced as compared with those of the corresponding controls, and in the two leg-bones they were almost obliterated so that the hypertrophic cartilage extended almost to the borders of the epiphyses. In the hypertrophic cartilage of the TA_3-treated rudiments, especially the femur, tibia and humerus, the intercellular partitions were narrower and more cells were degenerate than in the controls, but in the epiphyses of all five rudiments the cells were larger and the matrix more plentiful in the TA_3-treated explants. Periosteal ossification was proceeding actively in both series.

Comparison of the effects of TA_3 and T_3. Two experiments were made to compare the effects of TA_3 and T_3 in the same concentration (16 μg/100 ml. of medium), on the five limb-bone rudiments. One of each pair of bones was grown on medium containing TA_3 and the other in medium to which T_3 had been added.

No significant differences between the two series could be detected in gross anatomy, histological structure or growth in length (Fig. 1*B*); both sets of bones showed the changes described in the preceding section on the effects of TA_3 and in an earlier paper (Fell & Mellanby, 1956) on the action of T_3.

The behaviour of the explants in the presence of TA_4. Two experiments, exactly similar to those with TA_3, were made with TA_4. In a concentration of 16 μg/100 ml., TA_4 had no appreciable effect on either the gross structure or growth in length (Fig. 1*C*) of the rudiments; the explants were not examined histologically.

The behaviour of the explants in the presence of KI. Two control experiments were made to see whether potassium iodide (16 μg/100 ml. of medium) would affect the growth and development of the five rudiments. There was no significant difference in either the growth rate (Fig. 1*D*) or gross morphology of the two series. This result confirmed unpublished observations of the late Sir Edward Mellanby.

The metabolism of T_4 and T_3 by bones and medium

Thyroxine. In all experiments thyroxine and iodide were the only radioactive compounds detected in the bones or medium after incubation with ^{131}I-labelled T_4. The relative amounts of iodide and T_4 found in the different fractions and controls are shown in Table 1. Some spontaneous de-iodination of T_4 always occurred during storage. Although partial de-iodination by the medium was observed, it was considerably less than that produced by the bones. The proportion of the original thyroxine de-iodinated by the bones varied in each experiment, but could not be correlated with factors such as age of the embryo or length of culture period.

Triiodothyronine. T_3 and iodide were the only compounds detected in the bones or medium after incubation with ^{131}I-labelled T_3 (Table 1). T_3 underwent less spontaneous de-iodination than T_4, so that control values were always lower. De-iodination by the medium was only slight but a considerable amount occurred in the bones. The results of the two T_3 experiments were very similar despite the different culture periods, and although synthetically labelled T_3 was used in one experiment (no. 178) and biosynthetically labelled T_3 in the other (no. 204), this did not appear to have any effect on the results.

TABLE 1. Experiments with labelled compounds (marked *)

						Iodide formed (%)			
Expt. no.	Compound	Age of embryo (days)	Culture period	Exposure to T_4^* or T_3^* (hr)	Culture medium	Bones	Medium from bones	Medium alone	Iodothyronine alone
173	T_4	7	23 hr	23	Plasma + T_4 + concentrated embryo extract	69	40	38	17
176	T_4	7	20 hr	20	Plasma + T_4^* + dilute embryo extract	33	22	20	11
183	T_4	6	7 days	22	For 6 days, plasma + T_4 + concentrated embryo extract				
					7th day: (*a*) plasma + T_4^* + concentrated embryo extract;	(*a*) 37	24	22	—
					(*b*) plasma + T_4^* + dilute embryo extract	(*b*) 45	22	24	—
191	T_4 (syn)	8	21 hr	21	Plasma + T_4^* + concentrated embryo extract	52	40	41	40
178	T_3	7	19½	19½	Plasma + T_3^* + dilute embryo extract	38	9	6	6
204	T_3 (syn)	6	5 days	23	For 4 days, plasma + T_3 + concentrated embryo extract; 5th day, plasma + T_3^* + concentrated embryo extract	39	15	10	6

DISCUSSION

The results described above show that under the conditions of these experiments, the effect of TA_3 on the five limb-bone rudiments studied is quantitatively and qualitatively indistinguishable from that of T_3; earlier experiments (Fell & Mellanby, 1956) demonstrated that T_3 is roughly four times as potent as T_4 in reducing the growth rate of the femur and tibia in culture. In the same concentration TA_4 had no effect on the explants; whether this compound was indeed incapable of affecting the bones or whether it was rapidly broken down by the culture medium is not known.

The analytical results indicate that when embryonic bones are grown in the presence of T_4 or T_3, simultaneous partial de-iodination of the hormones takes place. T_3 was never detected in experiments in which T_4 had been used, although de-iodination had occurred. The de-iodination *in vitro* of T_4 by brain and muscle preparations without the production of significant amounts of T_3 has previously been described (Tata, 1957; Tata, Rall & Rawson, 1957); the present experiments substantiate the view that in many biological systems T_4 undergoes de-iodination without the production of detectable amounts of T_3. In our experiments iodide probably represents one of the end products of complete break-down of the hormones.

These results, therefore, provide no evidence for the hypothesis that T_4 owes its biological activity to a process of partial de-iodination, nor has the degradation of the alanine side-chain of the iodothyronine to the corresponding acetic acids been demonstrated.

SUMMARY

1. The investigation was undertaken to see whether limb-bone rudiments grown in culture in the presence of thyroxine (T_4) or triiodothyronine (T_3) produced any detectable transformation of the iodothyronine molecule.

2. T_3 and the iodothyroacetic acids were thought to be likely metabolites of T_4; the effects of these agents and also of potassium iodide on the growth of the explanted humeri, ulnae, radii, femora and tibiae of 6-day chick embryos were therefore studied, all the compounds being added to the culture medium in a concentration of 16 μg/100 ml.

3. Triiodothyroacetic acid (TA_3) diminished the growth of the tibia, femur and humerus in that order of degree, had little or no effect on the ulna and increased the growth of the radius; its effects were indistinguishable from those of T_3.

4. Tetra-iodothyroacetic acid (TA_4) had no appreciable effect on the rudiments.

5. In control experiments potassium iodide had no effect on the explants.

6. To investigate the effect of the limb-bones on T_4 and T_3, tibiae and femora from 6–8-day embryos were grown in medium containing ^{131}I-labelled T_4 and T_3; the break-down products of the hormones present in the explants, in the medium on which the rudiments had grown and in 'blank' medium incubated without explants were examined by chromatographic analysis.

7. T_4: at the end of the incubation period T_4 and iodide were the only radioactive compounds detected. T_3: only labelled T_3 and iodide were found.

8. The medium caused some de-iodination of T_4 but less than that produced by the explants; there was considerable de-iodination of T_3 by the rudiments, but very little by the medium.

9. These results support the view that in many biological systems T_4 is de-iodinated without the production of detectable amounts of T_3, TA_3 or TA_4.

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CONVERGENCE WITHIN THE PATHWAYS TO CAT'S SOMATIC SENSORY CORTEX ACTIVATED BY MECHANICAL STIMULATION OF THE SKIN

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The wide-spread cortical potentials evoked in the somatosensory cortex by stimulation of skin of a cat lightly anaesthetized with chloralose are in strong contrast to the highly localized responses in the animal anaesthetized with sodium pentobarbitone. Under chloralose not only are responses evoked in the 'association' cortex, but the primary somatosensory cortex is also more diffusely activated, so that the discreteness of the projections to somatosensory areas I and II may not be obvious. In addition, the cortical areas activated by stimulation of separated points in the peripheral skin overlap considerably.

This paper deals with experiments in which various factors were investigated which affected the recorded pattern of activation evoked in the primary somatosensory cortex by peripheral skin stimulation. In addition, the overlap of cortical areas activated by stimulation of separate points in the peripheral skin was studied to determine whether this spatial overlap was accompanied by convergence of pathways.

METHODS

Thirty cats were used; the majority were given intravenous chloralose (60 mg/kg body wt.) but a few were anaesthetized with sodium pentobarbital (35–40 mg/kg body wt.). The head of the animal was fixed in a stereotaxic head-holder and the lateral aspects of the frontal, parietal and temporal lobes were exposed on both sides. Cooling and drying of the cortex were prevented by covering it in a pool of paraffin saturated with Locke's solution and kept at 38° C with a heating ring. Mechanical stimulation of the skin was produced by a fine glass rod of diameter 0·5 mm with rounded tip attached to one corner of a Rochelle salt piezo-electric crystal (25 × 25 × 0·75 mm). By electrically pulsing the crystal with a square wave, control of onset, duration and amplitude of the mechanical displacement was attained (Gray & Malcolm, 1950). Maximum amplitude was approximately 0·25 mm; the duration was varied between 0·1–0·25 msec. Electrical stimulation of the cerebral cortex was effected by means of a bipolar platinum electrode isolated with an

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RF isolation unit. The interpolar distance was 2 mm. A monopolar wick electrode was used for surface recording on the cerebral cortex; the indifferent platinum electrode was in contact with the skin of the neck. For deep recording indium-filled glass micro-electrodes tipped with platinum and with an external tip diameter of 2–4 μ were used (Dowben & Rose, 1953). After amplification with an AC-coupled amplifier the action potentials were displayed on the face of the cathode ray tube and photographed on 35 mm film. Subsequent analysis of the records was made after projection in a photographic enlarger on squared paper. The microdrive used for traversing the cerebral cortex was a modification of the hydraulic microdrive described by Matthews (1952).

RESULTS

The cortical potentials evoked by mechanical stimulation of the skin of the contralateral limbs

The excitability of the pathways to the cerebral cortex for the afferent volley elicited by stimulation of the skin, and assessed by the complexity and distribution of the evoked cortical potential, was dependent on the type and depth of anaesthesia.

In cats anaesthetized with pentobarbitone sodium, monophasic or diphasic cortical potentials were evoked by maximal stimulation of the skin; the potentials were always initially positive and were recorded in two discrete areas, whose positions were dependent upon the contralateral peripheral point stimulated. These areas corresponded to those shown in the maps of Adrian (1941), and Marshall, Woolsey & Bard (1941).

In cats anaesthetized with chloralose a progressive increase in the amplitude of the peripheral stimulus to the skin from below threshold resulted always in the development of a diphasic evoked cortical potential in the primary somatosensory area, together with the increase in peripheral nerve action potential (Fig. 1). The potentials were always initially positive. The conduction velocity of the fastest fibres involved in the nerve action potential was 55–60 m/sec; this agrees with Zotterman's findings (1939) for the conduction velocity of the fastest fibres excited by tactile stimulation.

With a submaximal peripheral stimulus cortical potentials were evoked in two discrete areas similar to those obtained under pentobarbitone anaesthesia. This is shown in Fig. 2, in which the peripheral point stimulated was the contralateral forepaw. The areas also correspond to the somatosensory areas I and II of Marshall *et al.* (1941).

When the peripheral stimulus was increased to a supramaximal level a more wide-spread response was obtained. The complexity of the cortical response varied with the peripheral point stimulated. In Fig. 3*a* the map is shown of the evoked cortical responses to a supramaximal skin stimulation of the contralateral hind paw. The initially positive diphasic responses were limited to two discrete areas, but there was an adjacent wide-spread area of initially negative responses in the lateral and suprasylvian gyri not seen in animals anaesthetized with pentobarbitone. When the peripheral point of stimulation was the contralateral forepaw, increasing the stimulus amplitude led to an apparent fusion of the two areas with initially positive diphasic responses, while initially negative evoked potentials were again recorded in the lateral and suprasylvian gyri (Fig. 3*b*).

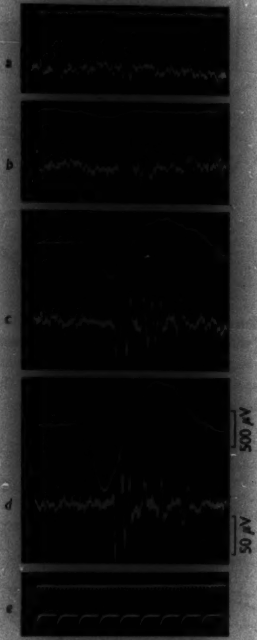

Fig. 1. Simultaneous records of the action potential in the intact sura nerve (lower record) and the evoked response in the contralateral somatosensory cortex (upper record) evoked by mechanical stimulation of the skin over the posterior aspect of the calcaneum. *a*, no stimulus; *b*, threshold stimulus for evoking the cortical response; *c* and *d*, stimulus strength progressively increased; *e*, time calibration (2 msec); upper trace time scale for cortical record and lower trace for peripheral nerve record. Stimulus indicated by vertical marker. Negativity of recording electrode for cortical response indicated by an upward deflexion.

In spite of the apparent fusion of the areas of the cortex where an initially positive evoked diphasic potential was observed, the dual nature of the projection to somatosensory areas I and II was maintained. This became evident when points on the cortex with the same latency for the beginning of the positive wave were joined by contour lines (Fig. 4). Fig. 4*a* shows the contour map obtained with a maximal stimulus to the forepaw. Two discrete areas of short latency, corresponding respectively to somatosensory areas I

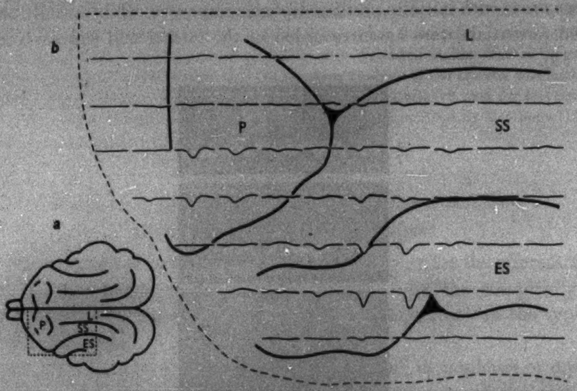

Fig. 2. Evoked cortical responses in a cat under chloralose anaesthesia on sub-maximal mechanical stimulation of the skin of the dorsum of the forepaw. *a*, dorsal view of cerebral hemispheres; *b*, enlargement of area in rectangle of *a*; P, L, SS and ES, post-sigmoid, lateral, suprasylvian and ectosylvian gyri respectively. The evoked responses shown were recorded at points 2 mm apart; upward deflexion recording electrode negative.

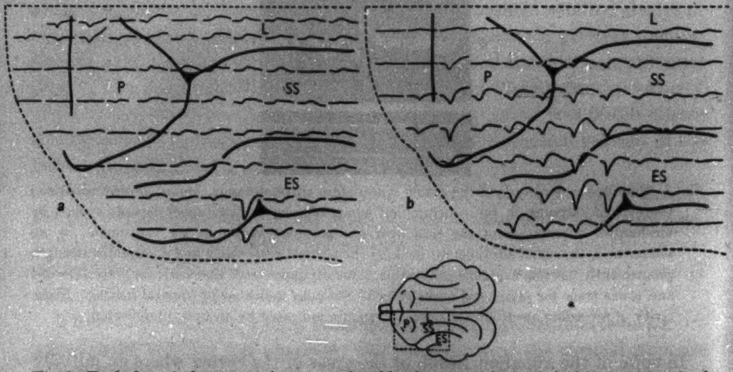

Fig. 3. Evoked cortical responses in a cat under chloralose anaesthesia evoked by supramaximal mechanical skin stimulation of the contralateral hind paw and forepaw. Inset, dorsal aspect of the cerebral hemispheres. *a* and *b*, enlargements of the area within the rectangle in inset: *a*, responses evoked by the stimulation of the contralateral hind paw; *b*, responses evoked by stimulation of the contralateral forepaw. The responses shown were recorded at points 2 mm apart. Upward deflexion recording electrode negative.

and II, are seen and in addition there is a third small discrete area of short latency in the anterior part of the suprasylvian gyrus. A corresponding area has been described by Marshall *et al.* (1941) in cats under pentobarbitone

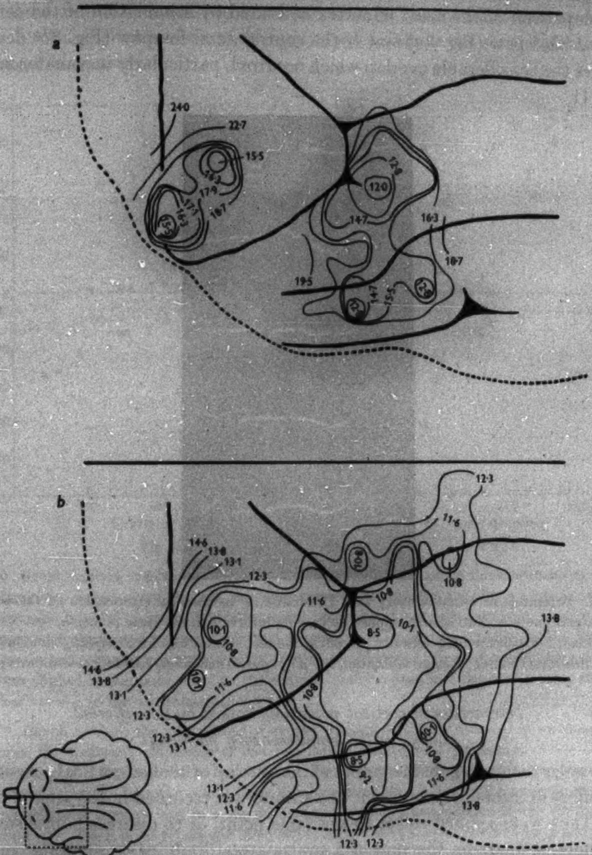
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Fig. 4. Inset, dorsal aspect of the cerebral hemispheres; *a* and *b*, enlargements of the area within the rectangle in inset. Points with the same latency for the beginning of the positive wave of the evoked response have been joined by 'contour' lines. This map has been constructed from the mean of eight observations made at points 1 mm apart on the cortex. Latency in msec. The peripheral point stimulated was the dorsum of the contralateral forepaw *a*, stimulus submaximal; *b*, stimulus supramaximal.

anaesthesia. When the peripheral stimulus to the contralateral forepaw was increased, the pattern of three islands of short latency persisted, although the latencies at all points was shortened by 4–6 msec. Thus the cortical activation, though wide-spread, is not indiscriminate.

Comparison of the areas of cortex activated by stimulation of the contralateral hind paw (Fig. 3*a*) and of the contralateral forepaw (Fig. 3*b*) demonstrates the considerable overlap which occurred, particularly in somatosensory area II.

Fig. 5. Potentials in somatosensory area II evoked by mechanical stimulation of the contralateral forepaw. Two stimuli applied at varying intervals to the same point. *a*, test response alone; *b*–*e*, conditioning stimulus preceding the test stimulus by an increasing time interval. Time scale 10 msec; voltage calibration 300 μV; upward deflexion recording electrode negative.

Interaction of cortical potentials evoked by two afferent volleys in the somatosensory pathways

In order to investigate the nature of the overlap of evoked cortical potentials, the effect of interaction between two stimuli was recorded in the area of overlap. They were applied either to the same point, or to discrete points several centimetres apart on the skin of a contralateral limb, or to separate contralateral limbs. The interval between the two stimuli was varied from 0 to 500 msec (Fig. 5). The result of interaction was not the same in the two primary somatosensory areas but was always in the nature of a block of the response to the second or test stimulus. No form of facilitation of the test stimulus was seen following any of the conditioning stimuli used.

In somatosensory area I, the greatest interaction occurred when the conditioning and test stimuli were applied to the same point on the skin. When the conditioning stimulus was applied to a different point but on the same limb, the interaction decreased as the distance between the two points increased, but some interaction still occurred even when the conditioning and test stimuli were applied to such widely separated points as the contralateral hind paw and forepaw.

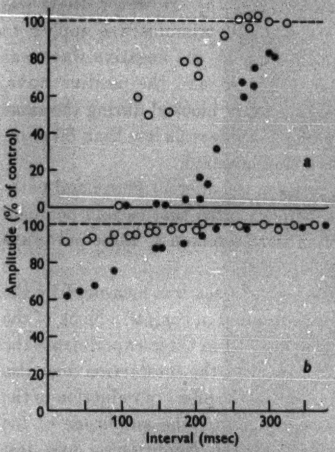

Fig. 6

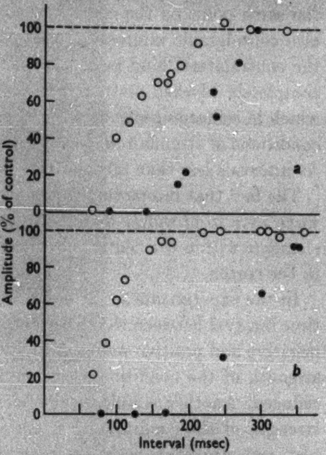

Fig. 7

Fig. 6. Graph to show interaction of evoked cortical potentials recorded in somatosensory area I. Ordinate, amplitude of the test response expressed as a percentage of the control; abscissa, stimulus interval in msec. Points represent the mean of eight observations. Positive wave amplitudes indicated by open circles and negative wave amplitudes by filled circles. Stimulus for the test response was applied to the contralateral forepaw. In *a* the conditioning stimulus was applied to the same point on the contralateral forepaw; in *b* the conditioning stimulus was applied to the contralateral hind paw.

Fig. 7. Graph to show the interactions of evoked cortical potentials recorded in somatosensory area II. Ordinate, amplitude of test response expressed as a percentage of the control; abscissa, stimulus interval in msec. As for Fig. 6 test stimulus was applied to the contralateral forepaw. In *a* the conditioning stimulus was also applied to the same point on the contralateral forepaw and in *b* the conditioning stimulus was applied to the contralateral hind paw. Each point represents the mean of eight observations. Positive wave amplitudes plotted with open circles and negative wave amplitudes plotted with filled circles.

The two graphs of Fig. 6 show the main features of these interactions. In both graphs the test stimulus was applied to the contralateral forepaw. In Fig. 6*a* the conditioning stimulus was applied to the same skin point. There was complete block of the negative wave (filled circles) at intervals of less than 200 msec and of the positive wave (open circles) at intervals of less than 80 msec. In Fig. 6*b*, the conditioning stimulus was applied to the contralateral hind paw. Even at the shortest intervals there was no complete block of the negative wave, but only some reduction of its amplitude. The positive wave was not significantly reduced even at the shortest intervals.

In somatosensory area II essentially the same result was obtained as in somatosensory area I when the conditioning and test stimuli were applied to the same point on the contralateral forepaw (Fig. 7*a*). However, there was also considerable interaction when the conditioning stimulus was applied to the contralateral hind paw. As is shown in Fig. 7*b*, the negative wave was completely blocked at intervals less than 170 msec, and the positive wave, which in somatosensory area I was not significantly blocked during the same conditions of stimulation, was greatly reduced at intervals less than 80 msec. At intervals less than 50 msec this wave was obliterated.

The fact that interaction occurred, even when the afferent peripheral pathways were from widely divergent points, demonstrated that certain neuronal elements within the central nervous system were common to these pathways to the cortex.

In the experiments so far described this convergence was measured by the time interval between the two stimuli which resulted in complete block of the negative and positive waves of the test response. If in these experiments the strength of the conditioning stimulus was reduced the interaction was also reduced. Another, simpler, way of demonstrating the effect of reduction in the strength of the conditioning stimulus was to measure the amplitude of the test response at a constant time interval between conditioning and test stimuli. The result of such an experiment is shown in Fig. 8. When a conditioning stimulus, which caused almost complete block of both waves of the test response (Fig. 8*b*), was reduced to less than half this strength, there was almost complete recovery of the positive wave and some recovery of the negative wave (Fig. 8*d*).

When reducing the interaction either by changing the strength of the conditioning stimulus, or by moving the point of application of the conditioning stimulus away from that of the test stimulus, the overlap of activated cortex produced by the separate stimuli also diminished, and this diminution paralleled approximately the reduction of the interaction.

Interaction of cortical potentials evoked within the callosal system

The experiments so far described demonstrate that there are common elements in the afferent pathways to the somatosensory cortex from widely separated peripheral points, but this gives no indication of the site of this convergence.

To examine the possibility of the cortex being the point of convergence, a second afferent cortical pathway, the transcallosal system, was investigated. This pathway has no anatomical overlap with the pathways activated by tactile stimulation, except in the cortex, as it has been shown by Curtis (1940*a*) that the responses evoked by stimulation of the opposite cortex are completely abolished by section of the corpus callosum.

Fig. 8. Cortical responses in somatosensory area II evoked by stimulation of the contralateral forepaw. Stimulus strengths indicated by the upward deflexion in the lower traces. Test stimulus was supramaximal and a control response is shown in *a*. In *b*, *c* and *d*, this was preceded by a conditioning stimulus at a constant time interval but with decreasing strength. Time calibration 10 msec; voltage calibration 300 μV; upward deflexion recording electrode negative.

Evoked cortical potentials mediated by the transcallosal system were produced by bipolar electrical stimulation of the symmetrically opposite cortex in the parietal lobe (Curtis, 1940*a*, *b*; Chang, 1953*a*; Peacock, 1957). Fig. 9 illustrates the effect of varying the stimulus strength. With a stimulus duration of 0·2 msec at low stimulus strengths the surface-evoked potential was diphasic and initially positive. With increase in the stimulus strength the amplitude of the initial positive and negative waves became greater and finally a later positive wave of long duration developed without further increase in the amplitude of the initial positive–negative complex. For the following experiments a stimulus strength was chosen which gave a maximum diphasic response like that seen in Fig. 9*c* without the late positive wave.

To investigate the pattern of interaction within the callosal system, two points 3 mm apart on the anterior suprasylvian gyrus were stimulated and the evoked potentials were recorded in the contralateral suprasylvian gyrus. This experimental arrangement is shown in the inset of Fig. 10. There was overlap of the areas of cortex activated by the two stimuli and recording was done in the area of overlap. One evoked potential was used to condition the test response following it and the interaction at varying time intervals between the stimuli was measured. Figs. 10 and 11 illustrate this interaction. Fig. 10 shows tracings of the evoked potentials obtained with five different intervals between the stimuli. The graph in Fig. 11 is constructed from a larger series of

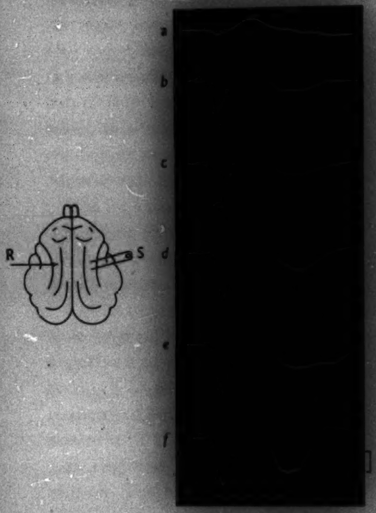

Fig. 9

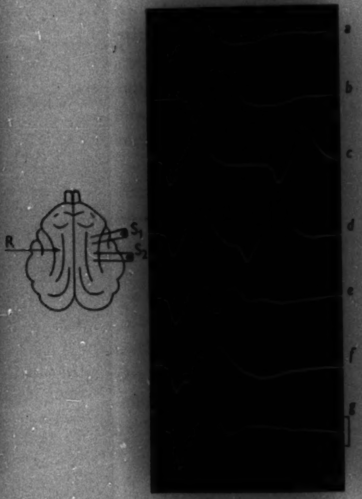

Fig. 10

Fig. 9. Cortical potentials recorded on the surface of the anterior suprasylvian gyrus (R) evoked by stimulation of the symmetrically opposite cortex using bi-polar electrodes (S). Duration of stimulus was 0·2 msec and stimulus strength was progressively increased from *a* to *f*; in *f* the late positive wave is seen. Time calibration 10 msec; voltage calibration, 500 μV; upward deflexion recording electrode negative.

Fig. 10. Cortical potentials evoked in suprasylvian gyrus (R in inset) by stimulation of contralateral gyrus at two discrete points (S_1 and S_2 in inset). *a*, Conditioning response; *b*, test response alone and *c*–*g*, interaction of these potentials with progressive reduction of stimulus intervals. Time calibration 10 msec; voltage calibration 500 μV; upward deflexion recording electrode negative.

similar records obtained in the same experiment. At short time intervals, where the two evoked potentials overlapped, the amplitude of the test response was determined by algebraic subtraction of the conditioning wave, as described by Chang (1953*b*). No blocking occurred at intervals longer than 50 msec.

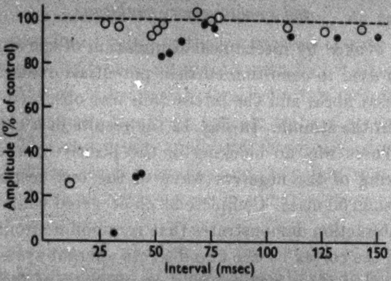

Fig. 11. Graph showing the interaction of cortical potentials evoked by transcallosal volleys arising from two discrete points on the contralateral cortex. Ordinate, amplitude of test response expressed as a percentage of the control. Abscissa, interval between conditioning stimulus and peaks of negative wave (closed circles) and positive wave (filled circles) respectively of the test response. Each point represents the mean of eight observations.

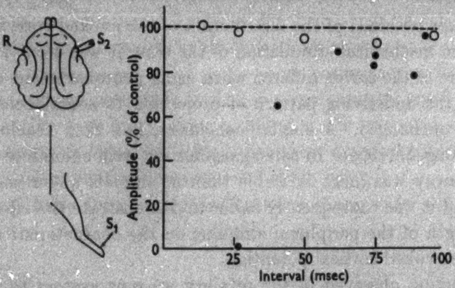

Fig. 12. Graph showing the interaction of evoked potential in somatosensory area II. The test response evoked by a transcallosal volley (S_2). Conditioning response evoked by mechanical stimulation of the skin of the contralateral forepaw (S_1). Ordinate, test response expressed as a percentage of the control amplitude; abscissa, interval in msec between the conditioning stimulus and the peaks of the negative wave (closed circles) and positive wave (filled circles) respectively of the test response. Each point represents the mean of eight observations.

Complete block of the negative wave occurred only when the interval between the stimuli was less than 25 msec, and at no interval was the positive wave completely blocked. Observations were not made at intervals of less than 10 msec. These results demonstrate that the recovery cycle of cortical neurones excited by a transcallosal volley is relatively short, and it is improbable therefore that these neurones are responsible for the longer-lasting block that occurs between volleys in the somatosensory pathways.

Interaction of cortical potentials evoked by volleys in the transcallosal and somatosensory pathways

The potential evoked by mechanical stimulation of the skin of the contralateral limb was used to condition cortical potentials evoked by stimulation of the transcallosal fibres and the interaction was observed at varying time intervals between the stimuli. In Fig. 12 the results of a typical experiment are displayed. There was no blocking of the positive wave of the callosal response. Blocking of the negative wave of the test response occurred at intervals of less than 50 msec. Complete block occurred when the interval was 25 msec. This interaction demonstrates that common neuronal elements were activated within the cortex by the two discrete afferent systems. In addition the results show that these cortical cells, at intervals of more than 50 msec following the arrival of a volley from the skin, had completely recovered their excitability and responded fully to a second afferent volley mediated by the callosal fibres.

DISCUSSION

From the maps obtained of the distribution of the evoked cortical potentials resulting from mechanical stimulation of the skin, it was seen that while the excitable state of the cortex differed when using pentobarbitone or chloralose anaesthesia, the underlying pattern of projection to somatosensory areas I and II was unchanged. A similar conclusion has been reached by Albe-Fessard & Rougeul (1955). In cats anaesthetized with chloralose the primary afferent pathway was more excitable than in animals under sodium pentobarbitone and it was consequently easier to demonstrate the effects of variation in strength of the peripheral stimulus on the characteristics and distribution of the evoked cortical potential.

The interaction observed in the primary afferent system to the somatosensory cortex on stimulation of separate skin points suggests that there are neuronal elements common to pathways from widely divergent peripheral points, such as forelimb and hind limb. In somatosensory areas I and II the degree of blocking between two peripheral volleys ran parallel to the degree of overlap of the areas of cortex activated by each of these volleys. The overlap of the two activated cortical areas and the degree of block between the volleys were changed simultaneously and in the same direction by varying the strength of the peripheral stimulus at one point.

The convergence which occurred within the somatosensory area I did parallel the overlap described by Gaze & Gordon (1954) as occurring within the nucleus ventralis posterior. This suggests that the convergence could occur within the specific afferent pathway but it does not indicate whether the convergence occurs at this synaptic relay or within the cortex itself. On the other hand, the convergence which occurred within the pathways to somatosensory area II was more extensive than the anatomical overlap in the thalamic relay nuclei described by Gaze & Gordon. As the cortical cells activated by a transcallosal volley in the somatosensory area II had regained their excitability within 50 msec after activation by an afferent volley from the skin, whereas the block between volleys from two peripheral skin points persisted for over 300 msec, the block appears to have occurred largely at a subcortical level. This conclusion applies to those cortical cells which were activated by both the transcallosal volley and the volley from the skin; it does not necessarily apply to all the neurones in somatosensory area II activated by stimulation of the skin.

There is the problem of whether the subcortical convergence between pathways from separate points on the peripheral skin to the somatosensory cortex occurs between the two specific afferent pathways or between a specific pathway and a less direct pathway to the cortex. If it were to occur between the specific afferent pathways to somatosensory area II then separate thalamic relays to somatosensory areas I and II would have to be postulated to account for the different degrees of convergence between pathways to these two areas. So far there is no convincing evidence for such separate relays, although Knighton (1950) has inferred their existence from experiments on stimulation of the nucleus ventralis posterior and a more posterolateral region, and by observing the cortical potentials evoked in somatosensory areas I and II.

The alternative possibility cannot, however, be excluded. The demonstration by Nauta & Whitlock (1954) that there are direct fibre connexions between the nucleus ventralis posterior and the intralaminar nuclei would offer an anatomical basis for convergence between the specific and non-specific pathways at this level.

SUMMARY

1. In cats anaesthetized with chloralose, the cortical potentials evoked by mechanical stimulation of the skin and by transcallosal stimulation were investigated.

2. Mechanical stimulation of the skin of the contralateral fore and hind paw evoked initially positive cortical potentials in somatosensory areas I and II. With supramaximal stimulation of the contralateral forepaw there was apparent fusion of these areas. However, when the latencies of the initially positive waves were mapped in contour the discreteness of the two areas was still evident.

3. When the skin was stimulated at points as widely separated as the contralateral forepaw and hind paw the areas of cortex activated in both somatosensory areas I and II overlapped.

4. Prolonged blocking interaction occurred between the cortical potentials evoked by stimulation of these widely separated skin points. This fact demonstrates the existence of neuronal elements within the central nervous system common to the two afferent pathways to the somatosensory cortex.

5. Blocking interaction of short duration occurred between potentials evoked by skin stimulation and by callosal stimulation. This is evidence for the convergence of the two systems within the somatosensory cortex. From the difference in the duration of the block seen with the two interactions, it is concluded that most of the long-lasting blocking interaction between pathways to the somatosensory cortex from separate skin points, and the underlying anatomical convergence, must be at a subcortical level. Further, this convergence probably occurs within the thalamus between the specific afferent pathway and less direct pathways.

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INTEGRATIVE PATTERN OF Ia SYNAPTIC ACTIONS ON MOTONEURONES OF HIP AND KNEE MUSCLES

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The importance of reflexes from muscles in regulation of posture and movement was demonstrated by the Sherrington school. The stretch reflex of Liddell & Sherrington (1924, 1925) was the basis of modern attempts to establish the contributions from the individual receptor systems in muscle. In 1946 Lloyd described the integrative pattern of the synaptic actions by large muscle afferents. From experiments on conditioning of monosynaptic reflexes to knee and ankle muscles it appeared that excitatory interconnexions were restricted to muscles operating synergically at the same joint (Lloyd, 1946; Laporte & Lloyd, 1952). Impulses in the same afferents were shown to cause an inhibition that was restricted to motoneurones of muscles with an antagonistic function at the same joint. With experiments that employed adequate stimulation (Lloyd, 1943) it was shown that the monosynaptic reflex formed the basis of the stretch reflex of Liddell & Sherrington (1924). Later it was demonstrated that the afferents concerned are those with the annulospiral endings on muscle spindles (Granit, 1950, 1955; Hunt, 1952; Laporte & Bessou, 1957; Eccles, Eccles & Lundberg, 1957*a*).

An extensive and quantitative investigation of monosynaptic interconnexions was made possible by the technique of intracellular recording (Eccles, Eccles & Lundberg, 1957*b*). Convergence of excitatory actions from various peripheral nerves to an impaled motoneurone was assessed from the size of the monosynaptic excitatory post-synaptic potentials (EPSP's) evoked by Ia volleys in various severed muscle nerves. In agreement with Lloyd (1946) and Laporte & Lloyd (1952) it was found that, for the majority of motoneurone species, the Ia receptiveness was restricted to muscles with a synergic action at the same joint. One notable exception was, however, regularly observed; the monosynaptic innervation from the knee extensor vasto-crureus to soleus motoneurones.

The present series of experiments was started in order to investigate the occurrence and significance of such interconnexions between muscles operating at different joints. Motoneurones to hip muscles proved to be particularly interesting from this point of view. The investigation was accordingly extended to a rather detailed survey of the convergence of excitatory and inhibitory afferents on motoneurones of hip and thigh muscles. On some motor nuclei extensive convergence has been found, with a pattern suggesting that, in some cases, the actions by I a impulses may subserve a co-ordinated reflex in response to movement of muscles operating at different joints. A preliminary report of some of the results has been given (Eccles & Lundberg, 1957).

METHODS

Cats were used, under light anaesthesia from pentobarbital sodium. The spinal cord was divided in the upper lumbar region, except in the experiments on motoneurones of the forelimb.

The recording techniques, methods of identification of motoneurones, and, most important, the method of assessment of the maximum monosynaptic EPSP's, are identical with those of Eccles *et al.* (1957*b*). A particular problem in this investigation was the necessity of using a number of ventrally situated nerves. For this purpose the thigh muscles were excised and the femur disarticulated at the hip and, in part, removed. The complete nerve to iliopsoas could not be dissected; the nerve used in this work was the main part of the nerve to the iliacus portion. It was mounted in a buried electrode between two heads of the muscle. The mounting had to be done carefully to prevent endangering the blood supply to the quadriceps nerve.

Micro-electrodes filled with 3 M-KCl were used throughout this investigation. When investigating inhibitory actions a depolarizing current (5×10^{-9} A) was passed through the electrode in order to prevent the IPSP's from reversing (Coombs, Eccles & Fatt, 1955). If the IPSP's were small or absent then the afferent pathway was subjected to a long train of impulses and the IPSP's were watched for in the post-tetanic period (Eccles & Lundberg, 1958).

RESULTS

Convergence of monosynaptic excitatory action

Hip flexors. Sherrington (1910) classified iliopsoas, sartorius and tensor fasciae latae (TFL) as hip flexors, both with regard to their mechanical action and their participation in the general flexion reflex. Amongst these muscles iliopsoas is the only one with an action at a single joint, the other two are double-joint muscles acting at the knee joint as well. Nevertheless, it will appear that the pattern of I a connexions to the motoneurones of these muscles is dominated by their hip flexor function.

The records in Fig. 1 were obtained from a sartorius motoneurone. The antidromic spike which is shown at threshold for the motor axon (*B*) and, at supramaximal strength (*C*), did not invade the soma. This neurone is I a receptive from the three hip flexors and from rectus, but not from any of the other muscle nerves (*G–M*). Table 1 shows that monosynaptic activations from rectus and from tensor fasciae latae on sartorius motoneurones are not consistent, the former occurring in 29 out of 54 neurones and the latter in

33 of 46, whereas the action from iliopsoas was found in all the 39 neurones tested. It is known that the sartorius muscle consists of two portions (Sherrington, 1910). The lateral is inserted mainly on the patella, and the medial, which is a smaller strip, on the tibia. In the dog these two strips are separated, but in the cat they are joined, although sometimes they are separately innervated, the medial portion receiving its innervation not directly from the femoral nerve but from the saphenous. Since contraction of sartorius in the cat leads not only to a hip flexion but also to a reduction of the femoro-tibial angle (Sherrington, 1910), the knee flexion by the medial strip is stronger than the knee-extensor function of the main part of the lateral band.

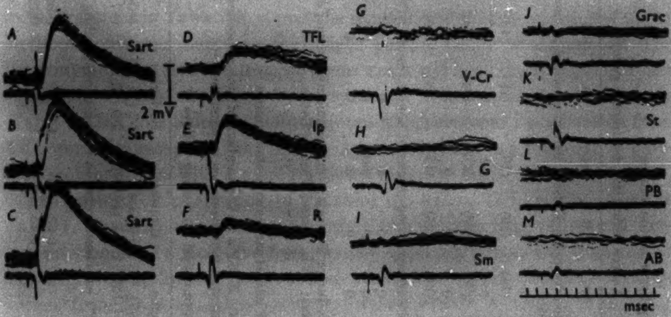

Fig. 1. Upper traces are intracellular records from a sartorius motoneurone, internal positivity (depolarization) being signalled upwards. Lower traces are recorded from the L6 dorsal root entry zone, negativity being signalled upwards. Records *A*, *B* and *C* show responses to shocks to sartorius nerve: *A*, below threshold for axon of this motoneurone; *B*, at threshold for this axon; *C*, above threshold for this axon. The antidromic impulse did not invade the soma. The remaining records show the effect of volleys in nerves to the muscles indicated in each record. Abbreviations: Sart, sartorius; TFL, tensor fasciae latae; Ip, iliopsoas; R, rectus; V-Cr, vasto-crureus; G, gastrocnemius-soleus; Sm, semimembranosus; Grac, gracilis; St, semi-tendinosus; PB, posterior biceps; AB, anterior biceps. All records were obtained at the same speed and at the same amplification.

In two animals with separate innervation of the medial and lateral bands the Ia receptiveness of motoneurones of these two divisions was investigated. Table 2 shows that only neurones innervating the lateral band are Ia receptive from rectus. Furthermore, the great majority of these neurones were also Ia receptive from TFL, whereas with the medial sartorius motoneurones this effect was small and infrequent. Another difference between these two subgroups is that medial sartorius motoneurones were Ia receptive from gracilis. Since in the one exceptional lateral sartorius motoneurone no EPSP's were evoked by volleys either from rectus or from TFL, it is assumed that this motoneurone actually innervated muscle fibres of the medial strip although its

TABLE 1. Hip flexor motoneurones

	Nerves stimulated for testing volleys											
Motoneurones	RP*	V-Cr	R	Add F	Sm	AB	Grac	St	PB	Ip	Sart	TFL
A. Mean resting membrane potentials and voltages or maximum monosynaptic EPSP's (mV) (numbers of motoneurones in which EPSP's were evoked and numbers of investigated motoneurones in parentheses)												
Iliopsoas	44·8 (20)	0 (18)	0·15 (8/18)	0 (14)	0 (14)	0 (16)	0 (16)	0 (18)	0 (18)	1·7 (21/21)	1·1 (21/21)	0·3 (8/16)
Sartorius	51·8 (56)	0 (58)	0·3 (29/54)	0 (28)	0 (38)	0 (38)	0·1 (7/38)	0 (34)	0 (34)	1·5 (39/39)	3·0 (58/58)	0·4 (33/46)
Tensor fasciae latae	56·5 (15)	0 (24)	0·4 (17/24)	0 (24)	0 (24)	0 (24)	0 (12)	0 (24)	0 (24)	0·3 (11/13)	0·8 (20/23)	2·8 (24/24)
B. Means of ratios of heteronymous EPSP's expressed relative to the corresponding means of homonymous EPSP's												
Iliopsoas	—	0	0·1	0	0	0	0	0	0	1	0·75	0·22
Sartorius	—	0	0·08	0	0	0	0·04	0	0	0·59	1	0·17
Tensor fasciae latae	—	0	0·18	0	0	0	0	0	0	0·17	0·3	1

* RP, Resting potential; V-Cr, Vasto-Crueus; R, Rectus; Add F, Adductor Femoris; Sm, Semimembranosus; AB, Anterior Biceps; Grac, Gracilis; St, Semitendinosus; PB, Posterior Biceps; Ip, Iliopsoas; Sart, Sartorius; TFL, Tensor Fasciae Latae.

TABLE 2. Medial and lateral sartorius motoneurones

A. Mean voltages of maximum monosynaptic EPSP's (mV) (numbers of motoneurones in which EPSP's were evoked and numbers of investigated motoneurones in parentheses)

	Nerves stimulated for testing volleys				
Motoneurones	Rectus	Gracilis	Iliopsoas	Sartorius	TFL
Med. sartorius	0 (0/14)	0·5 (6/7)	0·9 (4/4)	2·7 (15/15)	0·04 (3/10)
Lat. sartorius	0·4 (15/21)	0·02 (1/15)	1·1 (10/10)	2·9 (23/23)	0·7 (13/15)

B. Means of ratios of heteronymous EPSP's expressed relative to the corresponding means of homonymous EPSP's

Med. sartorius	0	0·13	0·63	1	0·02
Lat. sartorius	0·15	0·01	0·45	1	0·22

axon was in the nerve to the lateral portion. The I a receptiveness from gracilis may be considered in relation to the knee flexor functions of the medial sartorius, but it is noteworthy that these medial sartorius neurones are not I a receptive from the knee flexors, posterior biceps and semitendinosus. Although the main feature of the two subgroups to sartorius is their synergism with the hip flexor iliopsoas, it is clear that the different functions of the muscles are reflected by distinct characteristics in their I a receptiveness and also with respect to distribution of inhibitory action, as will be described below.

Table 1 shows that the motoneurones to iliopsoas are I a receptive from sartorius, TFL and rectus but not from any other muscle tested. With respect to the fractions in Table 1 *B*, it must be remembered that, as mentioned under Methods, only the nerve to the iliacus portion could be stimulated. The heteronymous innervation from sartorius was found in all the 21 motoneurones examined, whereas the effect from rectus and TFL was not found regularly (Table 1 *A*). Those iliopsoas motoneurones which received I a activation from TFL were also receptive from rectus. Apparently there is a subgroup of iliopsoas motoneurones with a more restricted receptive field.

The pattern of innervation to the third hip flexor, TFL, is also shown in Table 1. These neurones received a large homonymous innervation and were heteronymously I a receptive from the two other hip flexors and from rectus. The largest of the heteronymous actions was from sartorius, though the average actions from iliopsoas and rectus were also considerable. According to Sherrington (1910) TFL has two portions with somewhat different functions, the anterior longus portion with a pronounced hip flexor function and the posterior brevis in which abduction of the thigh predominates over the hip flexor function. The pattern of I a receptiveness has not shown two corresponding subdivisions such as is obtained with iliopsoas or sartorius. However, it is possible that our sampling of the TFL cells was not representative. According to Sherrington (1892) and Romanes (1953) this nucleus extends for several segments. Our standard procedure was to sample along the whole extent of any nucleus, but with TFL we had difficulties in finding the motoneurones except in the lumbar VII segments, where it lies slightly ventrolaterally to the semimembranosus nucleus.

Knee extensors. The finding that rectus afferents form part of the receptive field for the hip flexor motoneurones is of particular interest, because this muscle is I a synergic with the knee extensor vasto-crureus (Eccles *et al.* 1957 *b*). By synergism we imply that the rectus motoneurones receive I a impulses from vasto-crureus and that, conversely, vasto-crureus motoneurones are I a receptive from rectus.

Table 3 shows that the motoneurones to rectus are I a-activated from hip flexors as well as from knee extensors. Of the effects from the hip flexors the one from TFL is the largest (Table 3*A*), while reciprocally of the hip flexor motoneurones the TFL cells have the largest receptiveness from rectus (cf. Table 1). It is unlikely that the small and infrequent effects from sartorius and iliopsoas are of significance with respect to the reflex taxis of the limb, but they may be of significance in relationship to the problem of how the Ia connexions are established. With regard to the Ia effects from TFL and sartorius on rectus motoneurones it must be remembered that like rectus they are double-joint muscles with part of their insertion on the patella. It is therefore not surprising that they show some synergism with rectus. These connexions are, nevertheless, noteworthy because rectus is an extensor but TFL and sartorius are flexors, as evidenced by their participation in the general flexion reflex (Sherrington, 1910; Eccles & Granit, 1929) and by the synaptic actions of impulses in high threshold muscle afferents (unpublished observations).

The relative constancy of the interconnexions between the subgroups of quadriceps is well illustrated by the good agreement with the figures of Eccles *et al.* (1957*b*) and the present larger material in Table 3. For example, relative to the homonymous activation, the average rectus motoneurone had a Ia receptiveness from vasto-crureus of 0·24 (0·15 ± 0·09) and 0·23 in the two respective series. Motoneurones to vasto-crureus, on the other hand, are not Ia receptive from the hip flexors but receive Ia activation from the hip extensors, semimembranosus, adductor femoris and anterior biceps. These actions occurred in about half the neurones investigated (Table 3*A*), but the EPSP's were always small, the sum of average actions from the three hip extensors being smaller than that contributed by rectus (Table 3*B*).

Presumably these actions from hip extensors to vasto-crureus motoneurones are of little functional significance for the reflex activation of the muscle. Nevertheless, these connexions are of considerable interest in relation to the principles underlying the patterns of Ia receptiveness of motoneurones supplying muscles operating at different joints. The finding that soleus but not medial gastrocnemius motoneurones were Ia receptive from vasto-crureus raised the possibility that motoneurones to slow postural muscles could have a special pattern of reflex connexions (Eccles *et al.* 1957*b*). This hypothesis can now be tested on vasto-crureus motoneurones, since the origins and insertions of the slow crureus and the fast vasti are identical. In one experiment the convergence of Ia action from hip extensors to crureus and to vasti motoneurones was investigated separately; out of six identified crureus motoneurones none was Ia receptive from any hip extensor. Similarly, in another experiment four motoneurones which were identified as crureus on account of their long lasting after-hyperpolarization and the slow conduction velocity of their motor axon (cf. Eccles, Eccles & Lundberg, 1958) received no Ia activation from any of the three hip extensors. It can therefore be concluded that Ia receptiveness from muscles operating at a neighbouring joint is not a characteristic feature of motoneurones of the slow postural system.

Hip extensors. It was shown above that some vasto-crureus motoneurones receive a limited Ia excitation from the hip extensors. The converse situation is illustrated in Fig. 2, where a volley from vasto-crureus produced a substantial EPSP in a typical adductor femoris motoneurone, an effect that was regularly

Table 3. Knee extensor motoneurones

Motoneurones	Nerves stimulated for testing volleys											
	RP	V-Cr	R	Add F	Sm	AB	Grac	St	PB	Ip	Sart	TFL
A. Mean resting membrane potentials and voltages of maximum monosynaptic EPSP's (mV) (numbers of motoneurones in which EPSP's were evoked and numbers of investigated motoneurones in parentheses)												
Vasto-crureus	54·3 (32)	4·0 (44/44)	0·7 (39/44)	0·1 (21/43)	0·2 (24/44)	0·08 (18/43)	0 (0/41)	0 (0/42)	0 (0/42)	0 (0/6)	0 (0/41)	0 (0/22)
Rectus	56·8 (37)	0·9 (37/37)	4·0 (37/37)	0 (0/30)	0 (0/28)	0 (0/30)	0 (0/30)	0 (0/30)	0 (0/30)	0·02 (4/26)	0·1 (11/26)	0·4 (20/27)
B. Means of ratios of heteronymous EPSP's expressed relative to the corresponding means of homonymous EPSP's												
Vasto-crureus	—	1	0·15	0·05	0·09	0·06	0	0	0	0	0	0
Rectus	—	0·23	1	0	0	0	0	0	0	0·01	0·03	0·17

Abbreviations as in Table 1.

Table 4. Hip extensor motoneurones

Motoneurones	Nerves stimulated for testing volleys											
	RP	V-Cr	R	Add F	Sm	AB	Grac	St	PB	Ip	Sart	TFL
A. Mean resting membrane potentials and voltages of maximum monosynaptic EPSP's (mV) (numbers of motoneurones in which EPSP's were evoked and numbers of investigated motoneurones in parentheses)												
Adductor femoris	57 (27)	0·6 (29/30)	0 (30)	3·1 (33/33)	0·5 (28/32)	0·4 (26/30)	0·03 (5/27)	0·06 (6/11)	0·09 (5/11)	0 (12)	0 (24)	0 (15)
Semimembranosus	55·6 (55)	0·05 (16/82)	0 (80)	0·3 (37/58)	1·8 (81/82)	0·5 (79/82)	0·3 (42/57)	0·8 (67/76)	0·4 (67/74)	0 (15)	0 (80)	0 (39)
Anterior biceps	52·2 (11)	0·4 (14/32)	0 (32)	0·1 (7/12)	0·9 (26/30)	1·6 (35/35)	0·07 (1/11)	0·3 (11/33)	0·5 (22/30)	—	0 (26)	0 (15)
B. Means of ratios of heteronymous EPSP's expressed relative to the corresponding means of homonymous EPSP's												
Adductor femoris	—	0·27	0	1	0·21	0·16	0·01	0·03	0·06	0	0	0
Semimembranosus	—	0·03	0	0·25	1	0·31	0·17	0·43	0·21	0	0	0
Anterior biceps	—	0·29	0	0·05	0·63	1	0·05	0·33	0·38	—	0	0

Abbreviations as in Table 1.

observed (cf. Table 4*A*). The mean values of Table 4*B* show that this is the largest heteronymous excitatory action, and its relative size (0·27 of the homonymous) suggests that it has functional significance. Small EPSP's were also evoked by the nerves to the other hip extensors (Fig. 2 *C–E*) and also by posterior biceps semitendinosus (*F*).

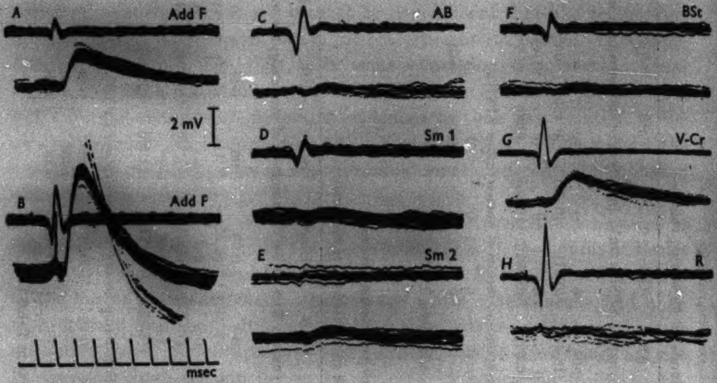

Fig. 2. As in Fig. 1, but for an adductor femoris motoneurone. Abbreviations additional to those given in legend to Fig. 1: Add F, adductor femoris; Sm 1, superficial head of semimembranosus; Sm 2, deep head of semimembranosus; BSt, posterior biceps + semitendinosus.

It may be asked if this contribution of Ia excitation by vasto-crureus is connected with the fact that adductor femoris, apart from its function as powerful hip extensor, is an adductor of the leg. In order to test this suggestion we recorded from motoneurones to a muscle with a pure adductor function, the adductor longus. In the single experiment of this type only one out of six adductor longus cells received a very small I*a* excitation from vasto-crureus. Thus it is likely that the Ia connexion from vasto-crureus to adductor femoris is related to the hip extensor function of the latter muscle.

The completely different receptive field of another hip extensor, semimembranosus, is illustrated in Fig. 3. Heteronymous EPSP's were here evoked not only from the nerves to the other hip extensors, adductor femoris (*C*) and anterior biceps (*D*) but also from the knee flexors, posterior biceps (*E*), semitendinosus (*F*) and gracilis (*G*). On the other hand, there was no Ia excitation from the vasti (*L*) nor from the crureus (*M*), such as was observed with adductor femoris motoneurones.

The knee flexors, semitendinosus and biceps, very frequently exerted a Ia excitatory action on semimembranosus motoneurones which together was almost as large as the homonymous excitation (Table 4). The large heteronymous action appears to be compensatory for the relatively small homonymous actions, as may be seen for example by comparison with adductor femoris motoneurones in Table 4.

In most experiments semimembranosus motoneurones had no Ia receptiveness for vasto-crureus, but in a few it has been common, giving the small mean effect of Table 4. There appears to be no difference in mechanical function of the two components of the semimembranosus muscle and both react in the same way in the flexion reflex (Sherrington, 1910). Usually there is independent innervation of these components so that it was possible to test their Ia distribution in two experiments. However, there was no significant difference.

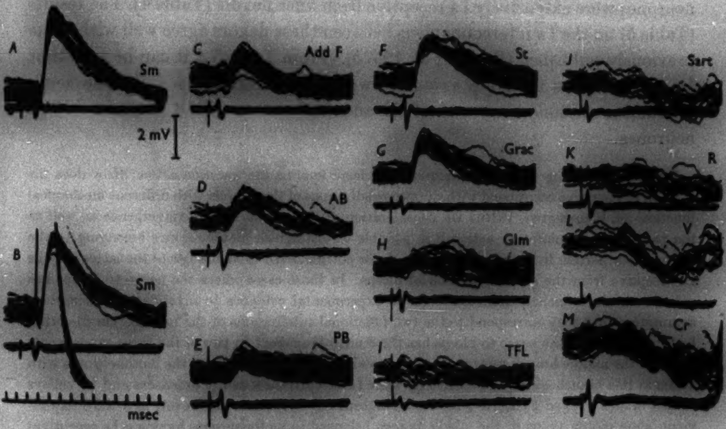

Fig. 3. As in Fig. 1, but for a semimembranosus motoneurone. Abbreviations additional to those given in the legend to Figs. 1 and 2: Glm, Gluteus medius; V, vasti; Cr, crureus.

Thus the receptive field for the two hip extensors, adductor femoris and semimembranosus, have displayed very distinct differences, adductor femoris receiving a substantial excitatory action from vasto-crureus and only a very small action from the knee flexors, whereas the receptive field of semimembranosus motoneurones includes the knee flexors but only occasionally vasto-crureus. The neurones of the third hip extensor, anterior biceps, seem to fall between these two types, with neurones showing a wider variation in their pattern of Ia innervation. Some anterior biceps motoneurones are Ia receptive not only from the synergic hip extensor semimembranosus and from the knee flexors semitendinosus and posterior biceps, but also from the vasto-crureus. Table 4*A* shows that 14 of the 32 anterior biceps neurones were Ia receptive from vasto-crureus. The actions from knee flexors did not occur as commonly as with semimembranosus (Table 4). The absence of effect from knee flexors was not correlated with a more frequent occurrence of excitatory action from vasto-crureus. Apparently there are two main subgroups within anterior biceps, those with and those without excitatory action from vasto-crureus. It is demonstrated below that neurones of the latter group are often inhibited by I a impulses from vasto-crureus.

Knee flexors. The monosynaptic interconnexion between the knee flexors semitendinosus, posterior biceps and gracilis was investigated in detail by Eccles *et al.* (1957 *b*). However, it is desirable to examine in addition the actions from the hip extensors, because reciprocally some of the hip extensor motoneurones were extensively I a receptive from knee flexors (Table 4). The results (Table 5) on the I a interconnexions between these flexors agree well with those previously published (Eccles *et al.* 1957 *b*). From Table 5 *A* it can be seen that I a receptiveness from hip extensors was rare, occurring for only one semitendinosus motoneurone out of 22 and for two out of 18 posterior-biceps neurones.

When investigating single motoneurones to biceps femoris the question arises: How does the division of the nerve correspond with the two subdivisions of this muscle with different mechanical function (cf. Sherrington, 1910)? On consideration of the monosynaptic convergence as well as the pattern of I a-inhibitory action it is possible to distinguish motoneurones belonging to the hip extensor portion from those to the knee flexor portion, even though a few of the latter may be I a receptive from the anterior portion of biceps. In those cases where the nerve to biceps has only two main branches, there has been no experimental evidence to invalidate Sherrington's assumption that they correspond to the two functional subdivisions of the muscle. On the other hand, in some cats the nerve to biceps has three main branches. In two of the three experiments in which the middle branch was mounted separately from the other two all the motoneurones with axons in this branch could be classified by their I a receptiveness as hip extensors and I a volleys in this branch evoked no EPSP's in semitendinosus motoneurones. However, in a third experiment some motor axons in the middle branch belonged to motoneurones having a I a receptiveness of knee flexor pattern and correspondingly the I a afferent fibres activated semitendinosus motoneurones. It is, therefore, advisable not to use the middle branch when discrimination between the extensor and flexor systems is required.

Forelimb motoneurones. Originally this work was started in order to establish the occurrence and significance of monosynaptic interconnexions between extensor muscles operating at different joints. As a link in this investigation the I a receptiveness of motoneurones to muscles in the forelimb was also explored. The monosynaptic interconnexions between the different elbow extensors were investigated by Eccles *et al.* (1957 *b*). Here the question of the I a receptiveness of these elbow extensor motoneurones from the shoulder extensor, supraspinatus, was studied.

Confirming Eccles *et al.* (1957 *b*), the homonymous EPSP on a motoneurone to the caput laterale of triceps brachii (Fig. 4) was relatively small and EPSP's of similar sizes were contributed from anconeus (*D*) and from the capita

TABLE 5. Knee flexor motoneurones

	Nerves stimulated for testing volleys										
Motoneurones	RP	V-Cr	R	Add F	Sm	AB	Grac	St	PB	Ip	Sart	TFL
A. Mean resting membrane potentials and voltages of maximum monosynaptic EPSP's (mV) (numbers of motoneurones in which EPSP's were evoked and numbers of investigated motoneurones in parentheses)												
Semitendinosus	58·2 (22)	0 (0/22)	0 (0/22)	0 (0/13)	0·05 (1/22)	0·2 (1/22)	0·6 (15/15)	3·7 (22/22)	1·3 (22/22)	0 (0/3)	0 (0/8)	0 (0/12)
Posterior biceps	61·4 (7)	0 (0/18)	0 (0/18)	0 (0/4)	0 (0/18)	0·06 (2/18)	0 (0/4)	1·7 (18/18)	2·6 (18/18)	— —	0 (0/8)	0 (0/8)
Gracilis	52·8 (13)	0 (0/18)	0 (0/18)	0·1 (3/17)	0·03 (2/15)	0 (18/18)	3·4 (18/18)	1·8 (18/18)		0 (0/3)	0·1 (2/10)	0 (0/3)
B. Means of ratios of heteronymous EPSP's expressed relative to the corresponding means of homonymous EPSP's												
Semitendinosus	—	0	0	0	<0·01	0·01	0·17	1	0·34	0	0	0
Posterior biceps	—	0	0	0	0	0·03	0	0·65	1	—	0	0
Gracilis	—	0	0	0·04	<0·01	0	1	0·6		0	<0·01	0

The large bracket signifies that the two nerves were used conjointly; abbreviations as in Table 1.

longum and mediale (*E*, *F*, respectively). In addition, a considerable EPSP was evoked by the volley in the nerve to supraspinatus (*C*). This monosynaptic connexion from supraspinatus was found with 21 out of 22 cells to the caput laterale, with 7 out of 8 cells to the caput mediale, and also with the three anconeus cells. By contrast, none of the 19 motoneurones to the double-joint portion of triceps brachii, caput longum, was found to be Ia receptive from supraspinatus. Although this pattern shows correspondence with what was found above for the different portions of quadriceps femoris, it is worth noting that the connexions in the forearm were found much more regularly and were also larger than those from the hip extensors to vasto-crureus. The mean EPSP evoked by a supraspinatus volley was of the same order of magnitude in motoneurones to the slow muscles, caput mediale and anconeus, as in those to the fast muscle, caput laterale. This finding provides further evidence that Ia receptiveness from muscles operating at a different joint is not necessarily prevalent with motoneurones to slow postural muscles.

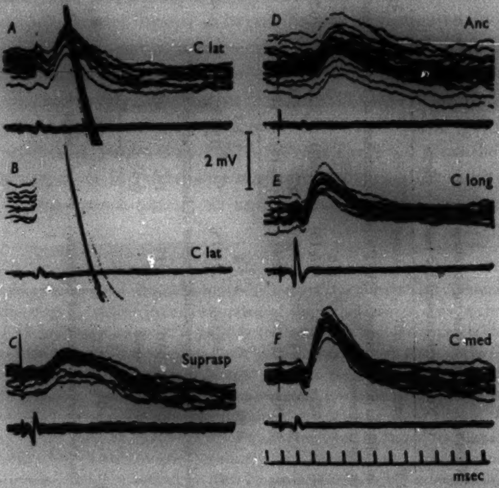

Fig. 4. As in Fig. 1, but for a forelimb motoneurone, caput laterale. Abbreviations are C lat, caput laterale; Suprasp, supraspinatus; Anc, anconeus; C long, caput longum; C med, caput mediale. All records at the same speed and amplification.

Convergence of inhibitory action

Hip flexor motoneurones. Since it is firmly established (Lloyd, 1946) that motoneurones are inhibited by Ia impulses from antagonist muscles, we should expect that the hip flexor motoneurones would be inhibited by Ia volleys from the adductor femoris, semimembranosus and anterior biceps nerves, as is actually shown in Fig. 5. The antidromic invasion in Fig. 5*A* identifies this motoneurone as belonging to iliopsoas. Record *B* shows the usual large heteronymous EPSP from sartorius. In *C*, *D* and *E* there were the expected IPSP's, which were produced by volleys in the nerves to adductor femoris, semimembranosus and anterior biceps. However, record *F* shows that an IPSP with a similar time course was evoked from the nerve to vasto-crureus, whereas stimulation of the rectus nerve gave only a late EPSP (*G*); neither was any IPSP evoked from the nerve to posterior biceps (*H*) or from any other nerve (not illustrated). The production of a Ia IPSP by vasto-crureus volleys was regularly found with other hip flexor motoneurones besides iliopsoas, i.e. with the motoneurones supplying sartorius and, less frequently, the TFL muscles. Fig. 5 further illustrates the characteristic feature that the IPSP from vasto-crureus was almost always larger than that evoked from any hip extensor nerve. Actually in many hip flexor motoneurones the IPSP from vasto-crureus was the only one observed, and hip extensor volleys produced IPSP's, if at all, only after post-tetanic potentiation.

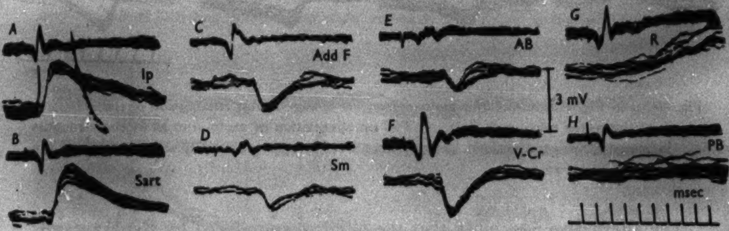

Fig. 5. As in Fig. 1, but for an iliopsoas motoneurone.

The latency and time course of all these IPSP's indicated that they were evoked by impulses in Ia afferents. An alternative possibility is that they are Ib IPSP's: but Ib IPSP's are not usually found in flexor muscles, only in extensors, and furthermore the time course of the Ib IPSP's is characteristically more prolonged than that of the Ia IPSP (Eccles, Eccles & Lundberg, 1957*c*). Since the ventral roots were intact so as to allow identification of the motoneurones, another possibility is that this inhibitory potential could be evoked by Renshaw cells activated by impulses in motor axon collaterals (Eccles, Fatt & Koketsu, 1954).

In order to differentiate between the Ia and Ib effects only animals with separation of the vasto-crureus volley into the Ia and Ib components were used (Bradley & Eccles, 1953; Eccles *et al.* 1957*a*; Laporte & Bessou, 1957).

In these cases the iliopsoas nerve was not prepared for stimulation, because this dissection may endanger the blood supply to the quadriceps nerve. It appears that in a sartorius motoneurone the IPSP is indeed evoked by a pure Ia volley in vasto-crureus nerve (Fig. 6*B*, *C* and *F*). By utilizing the double-volley technique the separation was shown to be complete at a stimulus strength of 1·45 times threshold (cf. Eccles *et al.* 1957*a*). In record *C* the IPSP was maximal at a strength of 1·45 times threshold. Finally, record *H* was obtained following post-tetanic potentiation and demonstrates that a small Ia volley of the same size as the one in *B* now gives a larger IPSP. It may be concluded that Ib impulses did not produce any of the IPSP in Fig. 6.

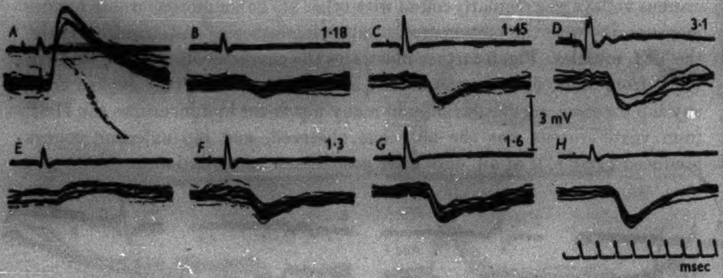

Fig. 6. As in Fig. 1 for a sartorius motoneurone. *A* obtained after stimulation of the sartorius, *E* of the rectus nerve and *B–D* and *F–G* on stimulation of vasto-crureus at the strengths indicated relative to threshold. Record *H* was obtained post-tetanically on stimulation of the nerve to vasto-crureus.

The alternative possibility that the IPSP was produced by Renshaw cells can also be excluded, because of the threshold differential between Ia afferents and the efferent alpha fibres. With carefully dissected nerves a maximum Ia volley can always be evoked by a stimulation subthreshold to the efferent alpha fibres. Fig. 7 shows the characteristic Renshaw IPSP in a rectus motoneurone. This neurone was found close by the sartorius motoneurone of Fig. 6. The IPSP appeared only when the stimulus strength was raised to 1·52 times threshold and was maximal at 1·80 in record *G*. That stimulation of efferent fibres in the vasto-crureus nerve was not the cause of the IPSP in Fig. 6 could be more directly excluded by the fact that in the vasto-crureus nucleus the field resulting from the antidromic invasion did not appear below a stimulus strength of 1·46. In another experiment it was shown that Ia volleys from vasto-crureus produced IPSP's in motoneurones that were located in the iliopsoas nucleus, but not specifically identifiable because the iliopsoas nerve was not prepared for stimulation.

The motoneurone in Fig. 6 innervated the lateral strip of sartorius, as evidenced by a monosynaptic EPSP from rectus in *B* (cf. Table 2). In Fig. 8 are shown intracellular records from a motoneurone to the medial strip of sartorius. The cell is identified by antidromic invasion by a medial sartorius volley (*A*), but receives its largest EPSP from lateral sartorius (*B*). EPSP's are also contributed by volleys from the nerve to iliopsoas (*C*) and gracilis (*D*), but not from TFL (*E*) and rectus (*I*). Instead the volley from rectus contributes an IPSP (*I*), which has the same time course as that from vasto-crureus (*H*).

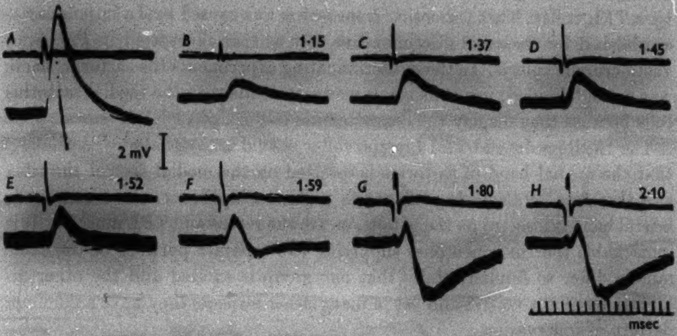

Fig. 7. As in Fig. 1, but for a rectus motoneurone. *A* was obtained after stimulation of rectus nerve and *B–H* following excitation of vasto-crureus at the different strengths indicated.

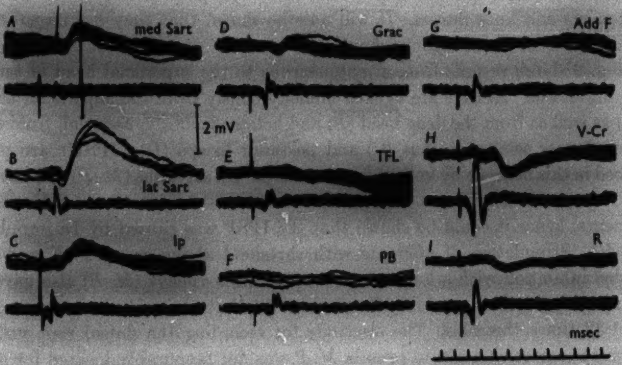

Fig. 8. As in Fig. 1, but for a medial sartorius motoneurone. Additional abbreviations are: med Sart, medial sartorius; lat Sart, lateral sartorius.

There is no Ia IPSP from adductor femoris (*G*), but in this animal none of the hip flexor motoneurones received inhibitory actions from the hip extensors. Usually IPSP's from volleys in hip extensor nerves were as frequently encountered in medial as in lateral sartorius motoneurones. In a few of the medial sartorius cells an IPSP with a similar time course as in *H* and *I* was also evoked by a TFL volley. That the action from rectus was caused by Ia impulses was established by the same discriminative test as that illustrated in Fig. 6 for vasto-crureus volleys. In these discriminating experiments the sartorius nerve was not subdivided; nevertheless, it was easy to identify the medial sartorius cells because they display the characteristic pattern of Ia EPSP's.

The IPSP produced by a Ia rectus volley should be correlated with the fact that the medial band of sartorius is inserted on the medial side of the tibia and therefore acts as a knee flexor as well as a hip flexor. In contrast the lateral band is inserted on the patella, as are the rectus and TFL muscles. It is remarkable that motoneurones supplying two separate portions of the same muscle differ so fundamentally that one group is excited and the other inhibited by rectus volleys and yet in many other respects they have a common pattern of innervation.

Hip extensor motoneurones. The distribution of inhibitory actions by Ia impulses to semimembranosus motoneurones was of particular interest because of the finding that they receive monosynaptic innervation from hip extensors and knee flexors. It will now be shown that they are maximally inhibited from the antagonistic muscles.

Fig. 9 shows records from a motoneurone to the superficial head of semimembranosus. EPSP's are evoked homonymously from the superficial head (*A*) as well as from the deep head (*B*) and heteronymously, as usual, from the knee flexors semitendinosus (*C*) and posterior biceps (*D*). IPSP's are produced in this neurone by volleys from rectus (*E–L*), sartorius (*M–P*) and vasto-crureus (*Q–S*). The rectus volley displayed separation into Ia and Ib components, hence it could be shown that the IPSP was caused by Ia impulses (*E–H*). Stimulation of afferents with threshold above group I (*K* and *L*) added inhibition with a longer latency. Sartorius volleys (*M–P*) also gave a similar IPSP, which was already maximal in *N* at the stimulation strength of 1·32 times threshold. The electrode for recording the dorsal root volley from the sartorius nerve in records *M–P* was not favourably located for disclosure of Ia–Ib separation, but with recording at a higher segmental level it was found that a stimulation strength at 1·32 times threshold evoked only the Ia volley. Finally, records *Q–S* show that a vasto-crureus volley evoked an IPSP with a similar latency and time course. This initial IPSP was already maximal with the small volley at 1·52 times threshold (*Q*). It was not possible to identify the volley responsible for the second phase of the IPSP with a latency of 3 msec (*Q*). The absolute threshold of the vasto-crureus nerve was higher than normal in this experiment and one possibility is that there was no discrimination between group I and II fibres; alternatively, the vasto-crureus volley in *Q* and *R* may have evoked a monosynaptic reflex which caused the late IPSP by the pathway of recurrent motor collaterals and Renshaw cells (cf. Eccles *et al.* 1954). In other experiments with threshold discrimination between the Ia and Ib volleys it was established that the IPSP from vasto-crureus was evoked by Ia impulses. Typical Ia IPSP's from rectus and sartorius are illustrated in Fig. 9, where the maximum IPSP from rectus (*I*) was larger than that from sartorius (*O*). The Ia IPSP from vasto-crureus was not observed as regularly as those from rectus and sartorius and was usually smaller. Volleys from the iliopsoas nerve usually produced in hip extensor motoneurones an IPSP of somewhat smaller size than that from sartorius, but there was rarely any effect from TFL.

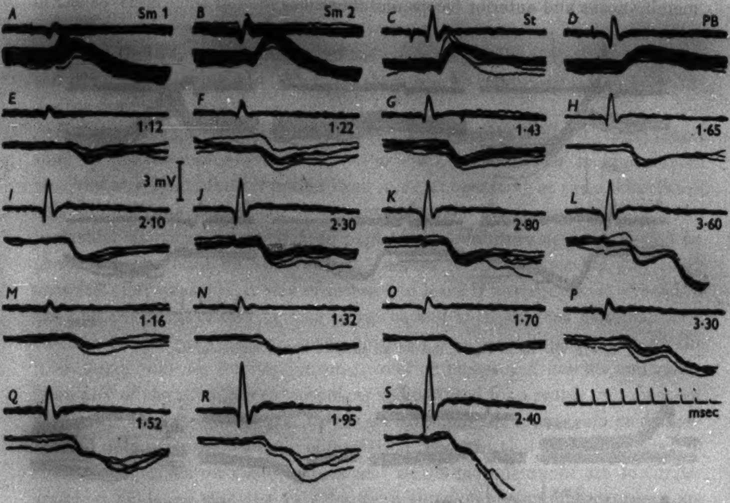

Fig. 9. As in Fig. 1, but for a semimembranosus motoneurone. *E–L*, *M–P* and *Q–S* are the effects produced by volleys in rectus, sartorius and vasto-crureus, respectively. The stimulus strengths relative to threshold of the respective nerve are indicated on the figure.

Fig. 10 shows records from an anterior biceps motoneurone with only a small homonymous EPSP (*A*), but with a large heteronymous EPSP from semimembranosus (*C*) and also a small EPSP from posterior biceps (*B*). The maximum Ia IPSP's evoked by rectus, sartorius and vasto-crureus volleys are shown in *D*, *E* and *F*. As observed with semimembranosus motoneurones the action from rectus is again the greatest. In those anterior biceps motoneurones which received monosynaptic excitatory action from vasto-crureus (Table 4) there was never any indication that they received Ia-inhibitory action from vasto-crureus as well. Hence, there are two groups of anterior biceps motoneurones, one with Ia-excitatory and the other with Ia-inhibitory action from vasto-crureus. The latter groups of neurones show a pattern of inhibitory actions similar to semimembranosus motoneurones. As shown in Figs. 5 and 6 the homonymous EPSP is often very small both with semimembranosus and anterior biceps motoneurones.

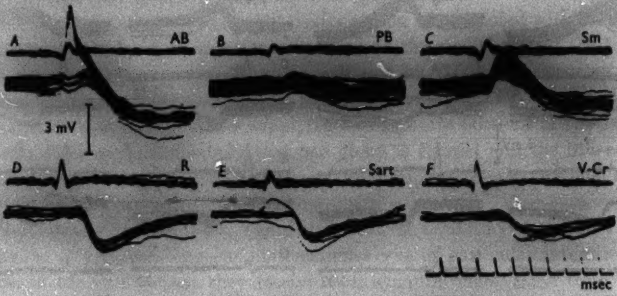

Fig. 10. As in Fig. 1, but for an anterior biceps motoneurone.

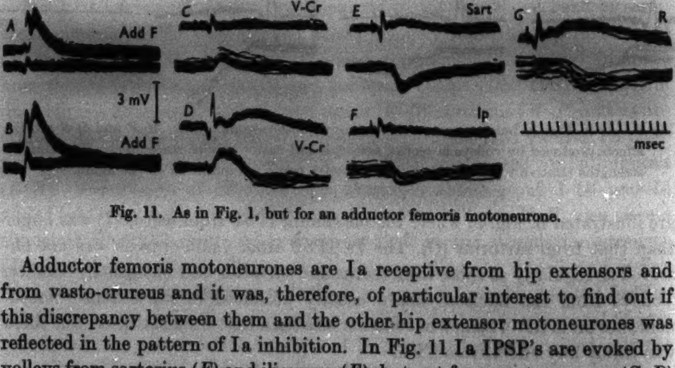

Fig. 11. As in Fig. 1, but for an adductor femoris motoneurone.

Adductor femoris motoneurones are Ia receptive from hip extensors and from vasto-crureus and it was, therefore, of particular interest to find out if this discrepancy between them and the other hip extensor motoneurones was reflected in the pattern of Ia inhibition. In Fig. 11 Ia IPSP's are evoked by volleys from sartorius (*E*) and iliopsoas (*F*), but not from vasto-crureus (*C*, *D*) or rectus (*G*). The late IPSP's in *D* and *G* were caused by impulses in group II afferents. Apparently the receptive field from which adductor femoris motoneurones draw their Ia inhibition was limited to iliopsoas, sartorius and TFL. Only in one cell was there a small Ia IPSP observed on stimulation of rectus.

Thigh adductors and abductors. Since the hip joint is a ball joint permitting movements in all directions it is possible to test if the Ia inhibition is organized exclusively to subserve extension and flexion or whether a similar linkage exists between muscles responsible for other reciprocal movement. Our findings indicate that this is not so between abductors and adductors of the thigh. In 13 motoneurones to adductor femoris investigated for effects from the abductor gluteus medius there was no trace of inhibitory action, nor did volleys in the nerve to gluteus medius or TFL evoke a trace of IPSP in any of the seven gracilis motoneurones investigated.

In the reciprocal situation no inhibitory actions were ever evoked in motoneurones to TFL on stimulation of the nerve to gracilis (15 cells). Only one cell to gluteus medius was found and there was no inhibitory action from adductor femoris or gracilis.

Knee extensors and flexors. Lloyd (1946) and Laporte & Lloyd (1952) found that neither the motoneurones to knee extensors nor those to the knee flexors received any Ia-inhibitory action from muscles operating at the ankle. Since this finding was confirmed in the investigation by Eccles *et al.* (1957*b*), particular attention has now been paid to the inhibitory actions exerted on these motoneurones by volleys in the nerves to the various hip and thigh muscles. For example, it was of interest to learn if any difference existed in the pattern of inhibitory actions to rectus and vasto-crureus motoneurones. Both these species of motoneurones, however, receive inhibition only from the knee flexor muscles semitendinosus, posterior biceps and gracilis and never from any of the three hip extensors, as is illustrated for a rectus motoneurone in Fig. 12. The effect from gracilis was always small and often could be demonstrated only after post-tetanic potentiation. The fact that the convergence of inhibitory actions on vasto-crureus and rectus motoneurones are so similar provides evidence that the Ia synaptic actions on rectus motoneurones are organized to subserve the knee extensor function. By contrast, the hip flexor function of rectus is illustrated by the distribution of the Ia actions from rectus. The actions of rectus and vasto-crureus volleys are qualitatively different on hip flexor motoneurones, whereas there are merely quantitative differences with respect to the action from these muscles on hip extensor and knee flexor motoneurones.

The motoneurones of the knee flexors, semitendinosus, posterior biceps and gracilis, receive inhibitory action from both vasto-crureus and rectus. The records in Fig. 13 were obtained from a gracilis motoneurone. It is characteristic of these three species of knee flexor motoneurones that the Ia IPSP evoked by a vasto-crureus volley (*I*) was somewhat larger than that from rectus (*H*), though the latter action was always significant. In particular, the IPSP's produced in semitendinosus neurones were often larger than those observed with any other species of motoneurone in the hind limb. Inhibitory actions by

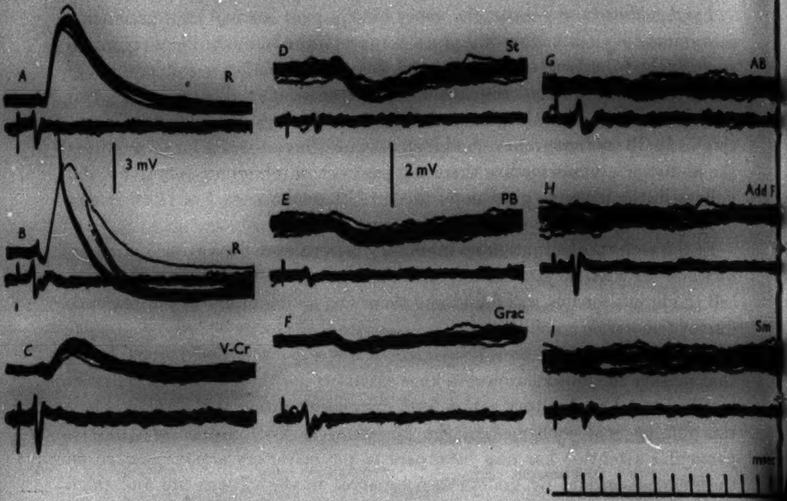

Fig. 12. As in Fig. 1, but for a rectus motoneurone. *A* and *B* were taken at the slightly lower amplifications indicated (3 mV), *C–I* at the 2 mV amplification.

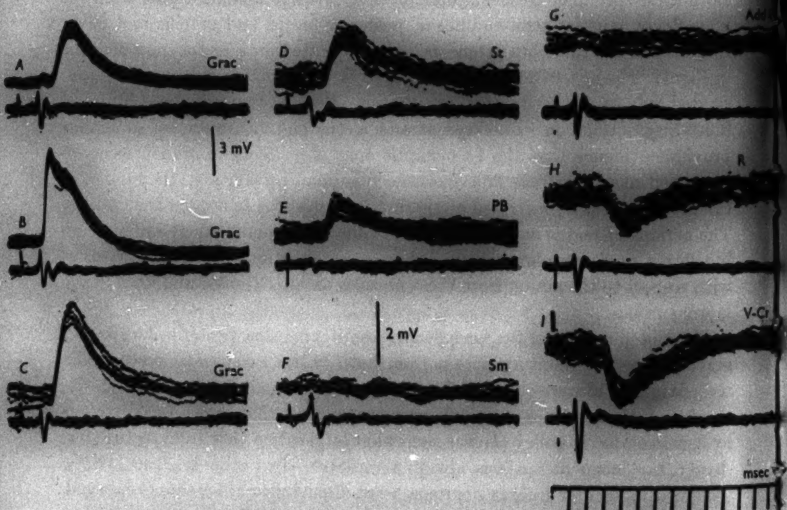

Fig. 13. As in Fig. 1, but for a gracilis motoneurone. *A–B* were taken at the slightly lower amplification indicated below record *A*; *C–I* at the higher amplification indicated above *F*.

Ia volleys from hip extensors and flexors were never observed in gracilis or posterior biceps motoneurones. However, in semitendinosus neurones Ia IPSP's were frequently evoked by a volley in the nerve to the lateral sartorius and occasionally also by a volley from TFL. No effect was ever found on stimulation of the nerve to iliopsoas. Rectus, TFL and lateral sartorius are all synergic double-joint muscles which are inserted on the patella; hence the inhibition from these three muscles is not surprising if it is considered that the double-joint muscle, semitendinosus, is their mechanical antagonist at both joints.

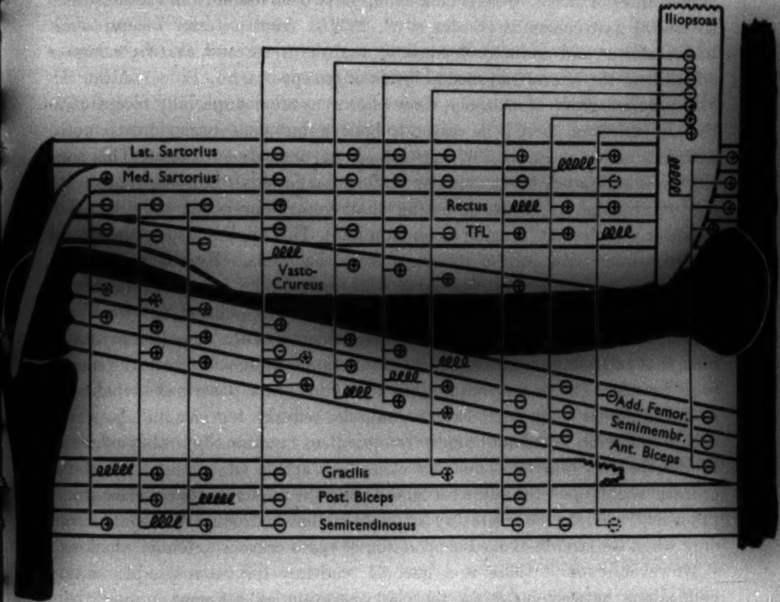
Iliopsoas
Lat. Sartorius
Med. Sartorius
Rectus
TFL
Vasto-Crureus
Add. Femor.
Semimembr.
Ant. Biceps
Gracilis
Post. Biceps
Semitendinosus

Fig. 14. Drawing summarizing the distribution of heteronymous excitatory actions ⊕ and inhibitory actions ⊖ evoked by impulses in Ia afferents from hip and knee muscles. Occasional or weak actions are denoted by hatched circles.

DISCUSSION

The present findings on the distribution of Ia-excitatory and Ia-inhibitory action on motoneurones of hip and thigh muscles are summarized in Fig. 14. The connexions are much more complex than those postulated by the hypothesis of the myotatic unit, which was formulated on the basis of experiments on reflex connexions to motor nuclei of knee and ankle muscles (Lloyd, 1946; Laporte & Lloyd, 1952). This hypothesis is therefore untenable as a generalization and in future investigations individual consideration must be given to each muscle or functional group of muscles.

A number of motor nuclei receive monosynaptic excitatory action from muscles operating at different joints. Although such connexions do occur between extensor and flexor muscles they are more common between extensor muscles. Strong connexions have been found from vasto-crureus to soleus (Eccles *et al.* 1957 *b*) and now from vasto-crureus to adductor femoris and to part of anterior biceps. Weaker connexions have been found from vasto-crureus to lateral gastrocnemius (Eccles *et al.* 1957 *b*), from anterior biceps, semimembranosus and adductor femoris to vasto-crureus, and also from supraspinatus to the lateral and medial heads of triceps brachii. Since, within the vasto-crureus group of neurones, those of crureus are not specially receptive to the hip extensors, there is no reason to believe that such connexions to motoneurones of the postural slow muscles are particularly prevalent. This conclusion is supported by the similar findings on forelimb motoneurones. On the other hand, it is worth noting that connexions occur only between extensor muscles not bridging a common joint. This applies also to the lateral gastrocnemius with part of its origin from the fibula. By contrast, motoneurones to the medial gastrocnemius, whose origin is entirely from the femur, were never Ia receptive from vasto-crureus. It was pointed out by Lloyd (1946), in the discussion of the general significance of the so-called myotatic unit, that two-joint muscles represented functional bridges between the single-joint muscles. The monosynaptic interconnexions now described would tend to link different single-joint extensor muscles reflexly, but this linkage which occurs on stretch would not be entirely equivalent functionally to that achieved by double-joint muscles. Thus, the excitatory action from stretch of vasto-crureus would affect the adductor femoris and the soleus motoneurones simultaneously, whereas the excitatory action to medial gastrocnemius would ensue only when the stretch-evoked contraction of vasto-crureus extended the knee.

However, even if there is a general tendency for monosynaptic interconnexions between extensor muscles operating at different joints, their functional significance should be considered mainly with respect to those motor nuclei that receive large actions from muscles operating at another joint. Presumably the existence of such connexions indicates that the Ia system to some motor nuclei is organized to achieve reflex activation through a complex movement. For example, the hip extensor, adductor femoris, will be maximally activated when both hip and knee extensors are stretched, i.e. by the combined movement of hip and knee flexion. Motoneurones of the hip extensor, semimembranosus, on the other hand, receive Ia excitation from hip extensors and knee flexors. Consequently, the most effective Ia activation in this case will be provided by the combined movement of hip flexion and knee extension. It should be considered in this connexion that the homonymous as well as the heteronymous action from other hip extensors on semimembranosus is small compared with most other species of motoneurones. Presumably the inflow from the knee flexors is required for proper I a-reflex activation of this muscle. The convergence of I a excitation on semimembranosus motoneurones is of particular interest because the receptive field consists of both extensor and flexor muscles. Apparently the organization of the I a system is not subordinated to the division into flexors and extensors.

The third hip extensor, anterior biceps, is transitional between semimembranosus and adductor femoris, with neurones displaying a less homogeneous pattern of receptivity. A number of them received considerable I a excitation from both vasto-crureus and the semitendinosus-posterior biceps. This would have been more puzzling without the experiments indicating the above two types of hip extensor motoneurones. However, there is even some overlap between these two types. Some adductor femoris motoneurones received small subsidiary excitation from semitendinosus-posterior biceps while semimembranosus cells were occasionally slightly I a receptive from vasto-crureus.

Corresponding with the differences in monosynaptic innervation of semimembranosus and adductor femoris there are characteristic dissimilarities in the distribution of I a-inhibitory actions to these motor nuclei. With semimembranosus the inhibitory pattern conforms strictly to the rule of reciprocity, since inhibitory actions were contributed by hip flexors, rectus and vasto-crureus. On the other hand, adductor femoris motoneurones usually received I a inhibition only from iliopsoas and sartorius. The excitatory action from vasto-crureus was not reciprocated by inhibition from the knee flexors. It should, however, be remembered that many adductor femoris motoneurones were actually slightly I a receptive from posterior biceps and semitendinosus, and it is possible that the occurrence of monosynaptic excitatory action from a certain muscle excludes the possibility of inhibitory actions from the same system of afferents. With some motor nuclei, amongst them anterior biceps, the neurones were actually found to receive either I a excitation or inhibition.

A clear exception to the rule of strict reciprocal innervation in the sense of the myotatic unit (cf. Lloyd, 1946, 1955; Laporte & Lloyd, 1952) is given by the I a inhibition from the pure knee extensor, vasto-crureus, to the motoneurones of the hip flexors. This inhibitory action was as a rule the largest received by the hip flexor motoneurones, but its functional significance is not clear. One possibility would be to classify it as a factor that, rather than representing a deviation from the concept of the myotatic unit, would restrict functionally the action by the I a system. Parts of the two hip flexor muscles, lateral sartorius and tensor fasciae latae, are double-joint muscles inserted on the patella; consequently, flexion at the knee will discharge I a impulses from them. From sartorius, in particular, large EPSP's are evoked in the hip flexor motoneurones, even in those of the single-joint muscle iliopsoas. On flexion of the knee the hip flexor motoneurones would thus receive excitatory action from sartorius, tensor fasciae latae and rectus, and inhibitory action from vasto-crureus. The latter effect may compensate for the former so as to limit the I a-excitatory action on hip flexors to those evoked exclusively from movements at the hip. However, the above reasoning is hypothetical; a more likely possibility to account for the inhibitory action from vasto-crureus is probably to assume that it has evolved in order to provide reflex actions that may assist locomotion (see below).

The present investigation on motoneurones to hip muscles has given abundant evidence of the remarkable correlation between muscle function and the pattern of I a linkages. No better example can be provided than the differences in I a connexions to motoneurones of the medial and lateral strips of sartorius in relation to the respective insertions of these two portions of the muscle. Both these subgroups of motoneurones receive a considerable I a-excitatory action from iliopsoas and also I a-inhibitory actions of the same order of magnitude from vasto-crureus. However, the lateral strip, with insertion mainly on the patella, has motoneurones which are I a receptive for excitation from rectus and tensor fasciae latae. By contrast, the motoneurones of the medial strip are I a receptive for excitation from gracilis, but receive I a inhibition from rectus. The I a-inhibitory action from vasto-crureus is shared by hip and knee flexor motoneurones, but the I a-inhibitory action from rectus is not shared with any other hip flexors, hence it must be associated with the knee flexor function of the medial strip of sartorius. Consequently, it appears that we have two distinct types of knee flexors, on one hand semitendinosus and posterior biceps and on the other medial sartorius. The functional independence of the medial sartorius motoneurones is demonstrated by the I a excitation from the hip flexors and I a inhibition from the hip extensors. Knee flexion will therefore occur when the medial sartorius contracts together with the other hip flexors in response to the movement of hip and knee extension. It is noteworthy that there are no monosynaptic interconnexions between the above two types of knee flexors. The fourth flexor, gracilis, gives some excitatory action to medial sartorius motoneurones, but the I a receptiveness of its own motoneurones shows clearly that this muscle is more related to posterior biceps and semitendinosus.

It is natural to inquire why there is such a complex organization of the I a relations between muscles operating at the hip and knee joints, when on the other hand, there are no I a connexions crossing the knee joint (cf. Lloyd, 1946; Laporte & Lloyd, 1952; Eccles *et al.* 1957*b*) apart from the single exception provided by the excitatory action from vasto-crureus to soleus (Eccles *et al.*

1957*b*). These differences become understandable if we assume that the Ia reflex actions have evolved to assist stepping. If in limb movement flexion occurred synchronously at all joints, and similarly with extension, there would be no need for any other organization than the myotatic unit. Philippson (1905) has made an analysis of the step in the dog's hind limb from films taken by Marey. As evidenced from changes of the femoro-tibial and tibio-tarsal angles, the synergic knee and ankle muscles contract synchronously. On the other hand, Philippson's analysis of the ischio-femoral angle indicated that neither the hip extensors nor the hip flexors contract synchronously with the other hind limb extensors and flexors. If we assume that these results also apply to the step in the cat, they could provide a basis for interpreting the more complex Ia linkage now found between the hip and knee muscles. A further evaluation of the relations between synaptic connexions of the Ia system and the sequence of muscular activation at the hip and knee joints during stepping is not possible solely on the basis of Philippson's analysis of angles, because it is hard to assess contributions from active and passive movements. For this purpose an electromyographic investigation would be needed. Such investigations have been made in the human (Eberhart & Inman, 1951), but not in the cat or dog.

The wide-spread convergence of Ia-inhibitory action on a number of motor nuclei merits consideration in relation to the interneurone in the inhibitory pathway (Eccles, Fatt & Landgren, 1956; Eccles & Lundberg, 1958). Hitherto this interneurone has been considered mainly as a commutator from an excitatory to an inhibitory pathway, but the question now arises, does it not also act as a simple integrative centre? It is not unlikely that this convergence occurs on the interneurones, and since an appreciable spatial facilitation has been demonstrated in the inhibitory pathway (Eccles & Lundberg, 1958) it is possible that during adequate activation the interneurones need this inflow from different sources to discharge effectively.

In the above discussion on the functional organization of the Ia system the results have been considered in relation to adequate activation through stretch of muscle. It is obvious (Eccles *et al.* 1957*b*) that the importance of the γ-system must also be discussed, since it is known that initiation of muscular contraction as well as maintenance of tonus may ensue through the γ-loop (Granit & Kaada, 1952; Eldred, Granit & Merton, 1953; Granit, 1955). At the time of these investigations as well as those on the reflex control of the γ-discharge (Hunt, 1952; Eldred & Hagbarth, 1954), there was general acceptance of the concept of the myotatic unit. It now appears that the distribution of Ia actions from some muscles is wide-spread and reaches motoneurones of muscles not immediately related in function. For example, vasto-crureus supplies monosynaptic excitatory action to rectus, soleus, adductor femoris and part of the anterior biceps motoneurones, and in addition Ia-inhibitory action to the hip flexor, the knee flexor and some of the hip extensor motoneurones. Hence, it is unlikely that the γ-loop is utilized for more precise movements. The wide-spread distribution of Ia-excitatory action is not prevalent with motoneurones to postural muscles, and therefore seems to be associated with the phasic rather than with the postural aspect of the Ia system. It has therefore seemed reasonable at this stage to discuss the new findings on the Ia pattern mainly in relation to activation through phasic stretch of muscles; but even so, it is clear that the γ-loop must be considered, since the sensitivity to stretch is set by the γ-system, and the γ-bias will presumably also decide how a contracted muscle is going to react to stretch.

SUMMARY

1. The pattern of Ia-excitatory and Ia-inhibitory actions to motor nuclei of hip and knee muscles has been investigated by intracellular recording from motoneurones. The excitatory interrelationship of shoulder and elbow extensors was also studied.

2. Reciprocal Ia inhibition is found between extensors and flexors, but not between adductors and abductors.

3. With a number of motor nuclei to hip muscles Ia-excitatory and Ia-inhibitory actions are organized in a more complex fashion than is postulated by the hypothesis of the myotatic unit.

4. Motoneurones of the hip extensor, semimembranosus, are Ia receptive for excitation from hip extensors and knee flexors, indicating that these motoneurones are most favourably activated by the combined movement of hip flexion and knee extension. Ia-inhibitory actions are contributed by all those muscles stretched by the reciprocal movement of hip extension and knee flexion.

5. Motoneurones of the hip extensor, adductor femoris, are Ia receptive for excitation not only from their own muscle but also from vasto-crureus, indicating that adductor femoris is most favourably reflexly Ia activated by the combined movement of hip and knee flexion. Inhibitory action is contributed only from hip flexors.

6. Within the hip flexor group strong synergism exists between sartorius and iliopsoas. With motoneurones of both these muscles the distribution of Ia actions has given evidence of functional subgroups. Two subgroups of sartorius motoneurones were correlated with the different mechanical functions of the lateral and medial strips of this muscle. Ia inhibition to hip flexors is provided not only from their antagonists but also from the knee extensor, vasto-crureus.

7. The functional significance of the Ia system is discussed mainly with regard to phasic reflex actions, which may assist locomotion. The complex

interconnexions between hip and thigh muscles in relation to the lack of such interconnexions between knee and ankle muscles may be correlated with the fact that, during stepping, movements in the hip and knee joints do not occur in phase as they do in the knee and ankle joints.

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THE EFFECT OF ATMOSPHERIC HUMIDITY ON THE SKIN TEMPERATURES AND SWEAT RATES OF RESTING MEN AT TWO AMBIENT TEMPERATURES

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The relationship to be expected between ambient humidity and skin temperature in the steady state, when other conditions are fixed, has been examined theoretically by Woodcock, Powers & Breckenridge (1956), who found it necessary to use two different methods of calculation according to whether the skin was completely wet with sweat or not.

In the case of completely wet skin, the rate of evaporation is determined by the air movement and the vapour pressure gradient between the skin surface and the air. For given circumstances, therefore, it is possible to calculate the skin temperature necessary for thermal equilibrium. Similar predictions of equilibrium skin temperature for circumstances in which the skin is completely wet have been put forward by Machle & Hatch (1947) and by Brunt (1947). These predictions are supported by published work relating to the determination of the hottest environments tolerable for long periods of time, if it be assumed that skin temperature is a primary limiting factor. They have also been confirmed in the case of resting men by a method depending on the examination of changes in skin temperature before equilibrium is reached (Kerslake & Waddell, 1958).

If the skin is not completely wetted by sweat, the rate of evaporative heat loss depends almost entirely on the rate of sweat production, and is found to be closely controlled at the level demanded by the ambient heat load and the metabolic rate. The only direct effect of ambient humidity in these circumstances is on the evaporative heat loss from the lungs and on the rate of diffusion of water through the skin. Since both these effects are small compared with the total heat exchanges in warm environment, it is to be expected that the rate of sweat production will be little affected by ambient humidity until the skin becomes completely wet. Robinson, Turrell & Gerking (1945) found a linear relationship between skin temperature and sweat rate when the metabolic rate was fixed, and by making use of their results Woodcock and his colleagues were able to predict a relationship of steady-state skin temperature and sweat rate to metabolic rate and ambient temperature, humidity and air movement. Hatch has also extended his analysis by a similar empirical assumption of a skin temperature: sweat rate relation, so that he is able to include circumstances in which sweat evaporation is complete.

These analyses lead to the expectation that if metabolic rate, air movement, wall temperature and air temperature are fixed, then, in the steady state, skin temperature and sweat rate will depend on ambient humidity. The relationship will be discontinuous since, below a certain critical humidity, at which the skin becomes completely wet, skin temperature and sweat rate will change only slightly for large changes in humidity, but above this level both will increase steeply as the humidity rises. The experiments described in the present paper were performed in order to check this prediction.

METHODS

Before an experiment the nude subject rested for half an hour in a room at 28° C, 40% relative humidity (r.h.) and 50 ft/min air movement. This induced mild thermal sweating He then moved into the test chamber and took his place on a deck chair which stood on the platform of a recording balance. The canvas of the chair was replaced by a wide-mesh nylon net, and a leg rest was provided so that the subject reclined with his lower legs horizontal and at about the level of the xiphisternum. Beneath the chair a metal screen was arranged so as to prevent dripping sweat from falling on the balance pan.

40 min after entering the test chamber the skin temperature was measured at 15 points (Kerslake & Waddell, 1958) and the mouth temperature was taken. The weight was recorded continuously over the next 20 min, at the end of which the temperature readings were repeated. Mean skin temperature is expressed as the mean of all the skin temperature readings, mouth temperature as the mean of the two mouth temperature readings and weight loss as the mean rate for the period of observation. In cases where sweat evaporation was complete, the weight record appeared linear, but at very high humidities the slope increased during the period of observation.

The constants for heat exchange by convection and radiation (k_{cr}) and by evaporation (k_e) had previously been determined using a dummy man in the position of the subject (Kerslake & Waddell 1958). They were respectively 8·9 ± 0·78 (S.E.) kcal/m^2.hr.°C and 7·8 ± 0·18 kcal/m^2.hr.mm Hg. Wall temperature was equal to air temperature in all cases. Air temperature and humidity were measured throughout the experiment by an aspirating psychrometer.

Calculation of sweat rate. Sweat rate was found from the recorded rate of weight loss by the subtraction of other sources of weight loss. The difference in weight between carbon dioxide produced and oxygen consumed was calculated from an assumed metabolic rate of 50 kcal/m^2.hr and a R.Q. of 0·85. The pulmonary water loss was calculated from the data of McCutchan & Taylor (1950), assuming a ventilation rate of 8 l./min. The allowance for insensible perspiration was based on the assumption that this is a diffusion of water vapour from the tissue fluid across the epidermis. The tissue fluid was assumed to be at skin temperature. The vapour pressure at the skin surface was found from the total rate of evaporation from the skin, the ambient water vapour pressure and the evaporation constant, k_e. The permeability of the epidermis was taken as 0·8 g/hr.mm Hg (Brebner, Kerslake & Waddell, 1956). The sum of all the calculated weight losses was about 40 g/hr in the driest conditions, becoming smaller as the humidity was raised. Total weight losses were at least 120 g/hr.

In cases where the rate of weight loss was changing during the period of observation, this rate does not accurately represent the sum of the rate of sweat production and the rate of weight loss from other channels, since in these cases sweat was dripping off the subject. It is to be expected that the amount of sweat lying on the skin will increase as the rate of dripping increases, so that the rate of change of weight will lag behind the actual rate of sweat production. Since such results do not, in any case, represent findings for the steady state, they require special interpretation, which will be elaborated when the results are described.

RESULTS

Two subjects have been examined at a dry-bulb temperature (D.B.) of 40° C and one of these has also been examined at 36° C. The results are presented in Fig. 1. Critical humidity is indicated by the vertical dotted line at about 33 mm Hg for 40° C and 37 mm Hg for 36° C. The sweat rate (○) appears to increase slightly as humidity rises from 10 mm Hg to the critical level, and to increase very steeply for higher humidities. The mouth temperature (●) behaves similarly. Skin temperature (◐) shows the same steep rise above the critical humidity, but below this level it does not seem to change with humidity.

It has been pointed out that the observations at high humidities were not made in the steady state. The weight record showed a progressive increase in the rate of weight loss during the period of observation, and both mouth and skin temperatures were rising. Previous examination of the situation when the skin is wet suggests that conventional calculation of the final equilibrium skin temperature may be made with some confidence (Kerslake & Waddell, 1958). Accordingly, the calculated curve for skin temperature in the steady state when the skin is completely wetted with sweat has been inserted in the figures. The observed points for wet skin lie below this line, since equilibrium had not been reached. The point at which the calculated temperature of completely wetted skin is the same as the observed temperature of incompletely wetted skin (the mean of the observations of skin temperature at humidities lower than the critical value) should represent the critical humidity. Points on the calculated line for wet skin below this humidity do not represent possible physiological states, since the rate of evaporation from completely wet skin in these cases exceeds the rate of sweat production to be expected at the skin temperatures concerned.

For conditions below the critical humidity, it is likely that the subject was near to the steady state. The rate of weight loss was approximately constant, and the skin and mouth temperatures showed little change between the beginning and end of the period of observation. The skin temperature usually rose about 0·1° C, but sometimes fell slightly, and the mouth temperature changed less than 0·1° C. If an increase in sweat production with skin temperature of 80 kcal/m^2.hr. ° C be assumed, the rate of heat loss from the skin will increase by about $(80+k_{cr})$ or 89 kcal/1° C rise in skin temperature. Allowing an increase of 3° C in skin temperature per 1° C rise in deep temperature as a conservative estimate for these conditions of low stress, and a

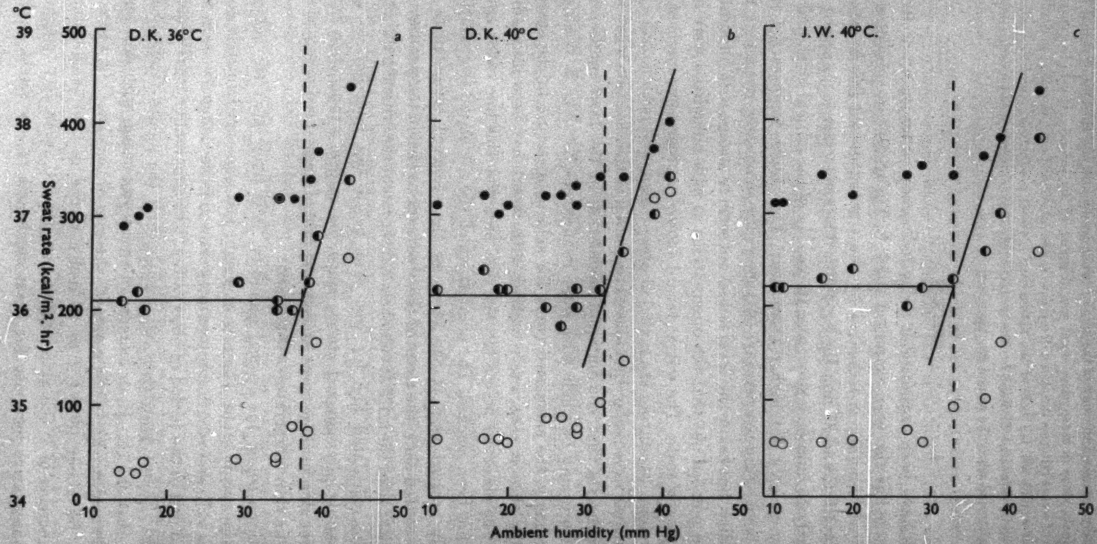

Fig. 1. Sweat rates, ○, skin temperatures, ◐, and mouth temperatures, ●, of nude resting subjects at various ambient humidities. The subject and the dry-bulb temperature, which is also the temperature of the surrounding surfaces, are indicated in each case. The sloping line indicates the calculated equilibrium temperature of wet skin. The horizontal line is drawn at the mean skin temperature for all observations at humidities below the critical level, which is defined by the intersection of these lines and indicated by the vertical dotted line.

surface:core heat-capacity ratio of 0·5, it would be expected that a given amount of stored heat would raise the skin temperature about twice as much as if the heat were uniformly distributed. On this basis, equilibrium would be 95% complete after 1 h. The skin temperature on entry was about 34·5° C and at the end of the hour about 36° C, so that the final observation should be within 0·1° C of the steady-state value. The observations suggest that the variability is greater than this, and that the error involved in assuming the steady state during the period of observation is small.

The increase in sweat rate with humidity below the critical value represents the change in the evaporative loss by channels other than sensible sweat production. Total evaporative heat loss did not change with humidity over this range, and the slope of the sweat-rate line is due to the corrections for water loss from the lungs and by insensible perspiration.

DISCUSSION

The results described are in agreement with the physical analyses referred to in the Introduction, and have some relevance to the problem of the classification of environments of equal heat stress. When, as in the present observations, metabolic rate may be regarded as constant, and the steady state only is under consideration, one must suppose that an increase in heat stress means an increase in the 'difficulty' of losing heat at the required rate. The physiological mechanisms which are called upon when the rate of heat loss would otherwise be reduced are (*a*) an increase in the thermal conductivity from core to surface, brought about by an increase in peripheral blood flow, and (*b*) an increase in sweat gland activity, which, if resulting in an increase in the rate of evaporation from the skin surface, will tend to reduce skin temperature and thus relieve the requirement for increased thermal conductivity. It may be concluded, therefore, that an increase in both skin temperature and sweat rate implies an increase in heat stress, as does also an increase in one of these quantities if the other is unchanged. It is impossible at present to comment on the nature of the change in heat stress should one increase while the other decreases, but in the results under consideration this did not occur. Results below the critical humidity are further simplified by the observation that the mean skin temperature for the subject D.K. was nearly the same for both environments (36·10° C at D.B. 36° C and 36·13° C at D.B. 40° C). In this range, while the sweat rate increased slightly with humidity at both dry-bulb temperatures, it was less at all humidities at D.B. 36° C than at D.B. 40° C. It would seem from the above considerations, therefore, that the heat stress was less at 36° C than at 40° C, irrespective of the humidity provided that this was below the critical value. The observations are plotted against Basic Effective Temperature (E.T.) in Fig. 2, from which it will be seen that, below the critical humidity, E.T. ranged from 27° to 33° C at D.B. 36° C and from

28° to 33° C at D.B. 40° C. The E.T. values thus do not seem to correspond to the heat stresses in the two situations. Above the critical humidity it is doubtful whether there is a significant difference between the sweat rates at D.B. 36° C and D.B. 40° C for the same E.T., although there seems to be a tendency for the former to be higher. The skin temperatures, however, are markedly different, being higher at D.B. 36° C than at D.B. 40° C for the same E.T. Since the skin temperatures are higher and the sweat rates are not lower, it may be concluded that above the critical humidity the heat stress for a given E.T. is higher at D.B. 36° C than at D.B. 40° C. Thus the E.T. scale seems inadequate as a description of environments of equal heat stress for the situation used in these experiments. There are several reasons why this might be so. The scale was designed for the case of men in shorts walking to and fro and is to this extent misapplied here. A more important consideration is that it was not derived from steady-state observations of physiological responses, but from subjective impressions on transfer from one environment to another. The effect of changes

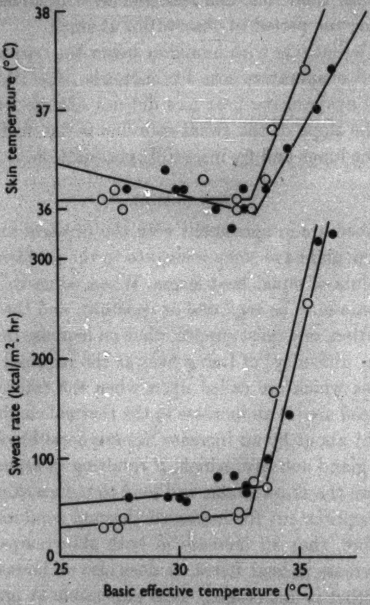

Fig. 2. Skin temperatures and sweat rates of subject D.K. plotted against 'Basic Effective Temperature'. ○, D.B. 36° C; ●, D.B. 40° C. The lines are intended to guide the eye and not necessarily to indicate significant differences.

in humidity below the critical level would be much more marked immediately after transfer than after prolonged exposure. The nature of the E.T. nomogram is such that it cannot show abrupt changes at the critical humidities for different environments, and since these changes are both to be expected on theoretical grounds and are found to occur in practice, the scale is likely to be misleading at high stress levels. This conclusion has previously been reached by Smith (1955) and by Lind & Hellon (1957).

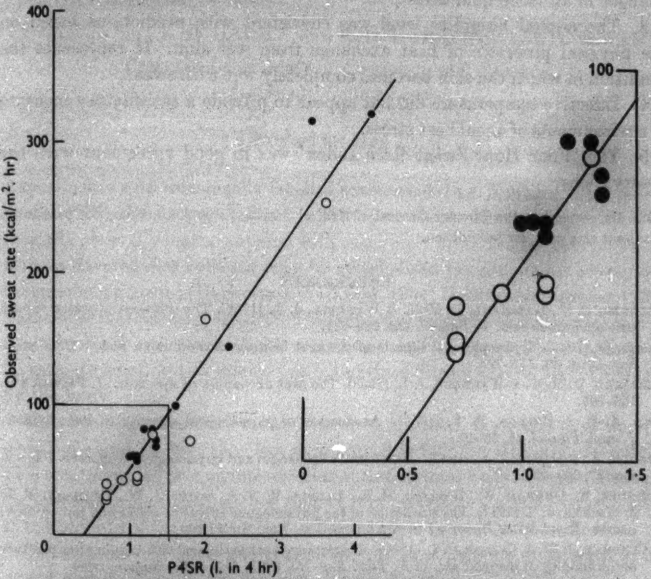

Fig. 3. Sweat rates of subject D.K. plotted against 'Four Hour Sweat Rate Index' (P4SR). ○, D.B. 36° C; ●, D.B. 40° C. Inset, lower values on larger scale.

The empirically derived 'Four Hour Sweat Rate' (P4SR) of McArdle, Dunham, Holling, Ladell, Scott, Thompson & Weiner (1947) is more successful in describing equivalence of the environments used for the present experiments. The observed sweat rates are plotted against the P4SR, derived from the published nomogram, in Fig. 3. It will be seen that there is no systematic difference between points relating to D.B. 36° C and those relating to D.B. 40° C, so that P4SR scale appears appropriately to describe environments which are equivalent in that they produce the same sweat rate response.

SUMMARY

1. Measurements of skin temperature, mouth temperature and sweat rate have been made on nude resting subjects at two dry-bulb temperatures and a wide range of humidities.

2. Humidity had little effect on the measured quantities below a critical value. Above this value, small changes in humidity were associated with large changes in all three quantities.

3. The critical humidity level was consistent with predictions based on the physical processes of heat exchange from wet skin. It represents the condition in which the skin becomes completely wet with sweat.

4. Effective temperature did not appear to provide a satisfactory measure of environments of equal heat stress.

5. The 'Four Hour Sweat Rate Index' was in good agreement with the observations.

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THE EFFECT OF A PROTEIN-FREE DIET ON THE COLLAGEN CONTENT OF MICE

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Experiments with isotopically labelled amino acids (e.g. Neuberger, Perrone & Slack, 1951; Neuberger & Slack, 1953) have shown that collagen is metabolically generally inert compared with other body proteins. In certain circumstances nevertheless collagen may be metabolized rapidly, as for example in the involuting uterus (Harkness & Harkness, 1954; Harkness & Moralee, 1956). These observations suggested that the reported inertness of collagen is in part dependent on stable metabolic conditions, and it appeared of interest to investigate whether this inertia was maintained during general loss of nitrogen by the body.

We therefore gave mice a diet free from protein. Over the experimental period of about 20 days they lost approximately half their initial body weight. This treatment led to a significant diminution in total body collagen, although the loss was proportionately less than that of protein as a whole. Examination of individual tissues showed that collagen was lost mainly from the skin.

Our experiments have also established the total amount of collagen in the mouse and its distribution between various tissues.

METHODS

Animals and diet. Adult male albino mice were used throughout. The protein-free diet contained 83% sucrose, 12% arachis oil, and 5% of the salt mixture described by Hubbell, Mendel & Wakeman (1937) with vitamin supplementation (Perez-Tamayo & Ihnen, 1953). The animals were allowed to eat and drink *ad lib.*

Experimental method. Two experiments were done, differing slightly in details. In each the animals were divided at the start into two equal groups by reference to a table of random numbers. Both groups were weighed and one (controls) killed with ethyl ether and stored at −10° C in sealed vessels. Experimental animals were given the protein-free diet and weighed on alternate days. They were killed and stored at −10° C for a few days before both groups were thawed and dissected for analysis.

In Expt. 1, twenty mice of a rather wide range of body weight were used. The experimental group was given the protein-free diet for 17 days. In all animals the abdominal viscera, excluding generative organs and kidneys, were weighed and analysed separately from the remainder of the body.

In Expt. 2, twenty mice were selected from stock in ten pairs, each pair having less than 1 g difference in weight between its two members. One member of each pair was then chosen at random to form the experimental group; the remainder were controls. The protein-free diet was given for 20 days. The body of each animal was analysed in five parts, each of which was weighed in a sealed bottle before analysis. First the two quadriceps muscles were removed. They were transected just above their patellar insertions and dissected free from their proximal attachments. Next the two femora were removed and scraped as far as possible free from ligamentous and muscular attachments. The skin was then removed, and last the abdominal viscera, excluding the reproductive organs and kidneys. The remainder, which contained the greater part of the muscle and bone of the animal, is referred to as the carcass.

Chemical methods. Collagen was brought into solution with hot trichloroacetic acid (TCA) (Fitch, Harkness & Harkness, 1955). In the first experiment the tissues were extracted twice with about ten times their volume of hot 5% (w/v) TCA and three times with cold TCA, soluble and insoluble material being separated by centrifugation. The tissues were homogenized in a small blender of the Waring type after the first heating with TCA. The combined extracts were made to a known volume. A sample was heated to dryness on a boiling water-bath and hydrolysed in 6 N-HCl for estimation of hydroxyproline by the method of Neuman & Logan (1950). In the second experiment, after the tissues had been heated in TCA the soluble and insoluble materials were not separated but an equal volume of 10% TCA was added to them. This brought the TCA approximately back to the original concentration since it was found that most of the original TCA had decomposed by this time. The material was heated for a further half hour and then made to a known volume. The suspension was mixed and a sample removed for estimation of total nitrogen by Ma & Zuzaga's (1942) modification of the Kjeldahl method. The remaining suspension was centrifuged at 2500 rev/min for 30 min, and portions of the supernatant were removed for estimation of hydroxyproline by Neuman & Logan's (1950) method, after evaporation to dryness on a boiling water-bath, and hydrolysis with 6 N-HCl in a sealed tube for 4 hr at 40 Lb./sq.in. (2·8 kg/cm^2) pressure in an autoclave. The collagen content of the tissue was calculated from the hydroxyproline content by multiplying by a factor of 7·46. Non-collagenous nitrogen was calculated by deducting collagenous nitrogen from the total.

RESULTS

The results of the first experiment are given in Table 1. The animals of the experimental group lost about 45% of their initial body weight during 17 days on the protein-free diet and the total collagen content of their bodies was 87% of that of the control animals. This difference was significant when tested by analysis of co-variance to eliminate the effect of difference in initial body weight between the two groups $(0{\cdot}01 > P > 0{\cdot}001)$. The abdominal viscera of the deficient animals also contained less collagen than the controls, but the difference was not significant $(P < 0{\cdot}05)$.

TABLE 1. Collagen content of control mice and mice fed on protein-free diet (Expt. 1)

	Body wt. at death (g)	Total collagen (mg)	Skin and carcass collagen (mg)	Visceral* collagen (mg)
Control	35·0 ± 1·2	1000 ± 40	980 ± 70	23·6 ± 1·6
Protein-free diet	19·7 ± 2·2	870 ± 30	850 ± 20	20·1 ± 0·8

* Abdominal viscera excluding kidneys and genital organs.
The estimate of variation is the standard error of the mean.

The results of the second experiment are given in Tables 2–4. The experimental diet produced a significant loss of total body collagen ($0{\cdot}01 > P > 0{\cdot}001$). The loss (11 %) was less than the loss in body weight (44 %) or non-collagenous N (33 %). Collagen formed 2·6 ± 0·1 % of the body weight and 17·7 ± 0·7 % of total N in the control animals.

TABLE 2. Weight, collagen and non-collagenous nitrogen (NCN) in different parts of the body in normal mice and mice fed on a protein-free diet (Expt. 2)

	Controls					Protein-free diet				
	Wet wt. (g)	Total collagen (mg)	Collagen as % of wet wt.	NCN (mg)	NCN as % of wet wt.	Wet wt. (g)	Total collagen (mg)	Collagen as % of wet wt.	Total NCN (mg.)	NCN as % of wet wt.
Skin	6·67	442*	6·7	216*	3·23	3·96	369	9·3	171	4·35
	± 0·17	± 26	± 0·4	± 8	± 0·08	± 0·11	± 26	± 0·6	± 20	± 0·13
Carcass	24·11	539	2·20	503*	2·09	14·03	496	2·90	327	2·34
	± 0·37	± 12	± 0·06	± 22	± 0·06	± 0·48	± 51	± 0·08	± 14	± 0·08
Viscera	7·53	32	0·50	159	2·15	3·67	29	0·80	91	2·53
	± 0·38	± 1	± 0·03	± 18	± 0·12	± 0·12	± 1	± 0·03	± 8	± 0·15
Femora	0·160	15·1†	9·40	2·3†	1·45	0·170	15·6	8·9	2·8	1·66
	± 0·005	± 0·5	± 0·35	± 0·4	± 0·09	± 0·006	± 0·8	± 0·5	± 0·6	± 0·16
Quadriceps	0·53	4·3	0·81	18·0	3·38	0·29	4·1	1·45	10	3·57
	± 0·02	± 0·1	± 0·02	± 0·6	± 0·07	± 0·02	± 0·1	± 0·05	± 0·7	± 0·21
Total	39·00	1028*	2·6	893*	2·26	22·12	913	4·2	602	2·49
	± 0·54	± 25	± 0·11	± 36	± 0·08	± 0·64	± 33	± 0·3	± 21	± 0·22

The estimate of variation is the standard error of the mean.

* Nine animals only, one analysis of skin being lost accidentally.

† Seven animals only, three analyses being lost accidentally. For computation of total body collagen figures were estimated for femora from their wet weight and percentage collagen in other animals. The femora formed such a small proportion of the total (1·5 %) that no appreciable error is introduced by this procedure.

TABLE 3. Distribution of weight, collagen and non-collagenous nitrogen (NCN) in bodies of control normal mice and mice fed on a protein-free diet (Expt. 2)

	Controls			Protein free diet		
	Wet wt. as % of total wet wt.	Collagen as % of total collagen	NCN as % % of total NCN	Wet wt. as % of total wet wt.	Collagen as % of total collagen	NCN as % of total NCN
Skin	17·11	42·71	24·65	17·92	40·06	28·46
	±0·46	±1·76	±0·81	±0·37	±1·76	±0·79
Carcass	61·84	52·31	55·25	63·37	54·54	54·54
	±0·63	±1·72	±1·33	±0·46	±1·61	±1·06
Viscera	19·27	3·11	17·79	16·59	3·24	15·26
	±0·86	±0·13	±0·94	±0·43	±0·21	±0·62
Femora	0·42	1·49	0·27	0·79	1·71	0·47
	±0·01	±0·06	±0·02	±0·02	±0·08	±0·05
Quadriceps	1·43	0·41	1·99	1·31	0·45	1·70
	±0·05	±0·01	±0·07	±0·03	±0·02	±0·05

The estimate of variation is the standard error of the mean.

The results of analyses of different tissues showed that some lost a greater proportion of their collagen than others. No loss of collagen was found in bone, as judged from the femur. The collagen content of the quadriceps muscles was only 5 % lower in the experimental mice, a difference which was not significant. The collagen content of the carcass was however significantly lower ($0{\cdot}05 > P > 0{\cdot}02$) in the experimental animals. There is some apparent anomaly in the fact that while the carcass showed a significant reduction in deficient animals two of its major constituents, bone and muscle, did not. It is obvious that the carcass is in effect a collection of units similar in type to quadriceps muscle and femur. Part of the explanation may lie in experimental error in measuring comparatively small amounts of collagen.

The difference between the mean collagen content of the viscera of the control and experimental groups was not significant. However, the fact that a similar but again just not significant difference was found in the first experiment suggests that the viscera do in fact participate in the general loss of collagen. The largest significant difference (16 %) in the collagen content between the control and experimental animals was found in the skin ($0{\cdot}05 > P > 0{\cdot}02$).

TABLE 4. Losses of wet weight, collagen and non-collagenous nitrogen calculated from difference between means for experimental and control groups (Expt. 2)

	Losses, absolute and as % contribution to total loss			Percentage difference, control mice minus experimental mice		
	Weight (g)	Collagen (mg)	NCN (mg)	Weight	Collagen	NCN
Skin	2·71 (16·0 %)	72·6 (61·4 %)	45·0 (15·3 %)	40·6	16·4	20·8
Carcass	10·07 (59·6 %)	43·0 (36·3 %)	176·1 (59·9 %)	41·8	7·9	34·9
Viscera	3·87 (23·0 %)	3·0 (2·5 %)	65·1 (22·1 %)	51·4	9·3	41·6
Femora*	−0·0099 (−0·05 %)	−0·0005 (−0·4 %)	−0·41 (−0·1 %)	−6·0	−3·0	−2·7
Quadriceps	0·24 (1·4 %)	0·2 (0·1 %)	8·1 (2·7 %)	45·1	3·7	18·9

* Negative values indicate apparent gains by experimental group.

The relative importance of losses from the different parts of the body may be demonstrated by expressing the loss from each part as a percentage of the loss for the whole body (Table 4). Whereas the greatest part of the loss of weight and non-collagenous nitrogen (59·7 and 59·9 % respectively) was from the carcass, the greater part (60 %) of the loss of collagen was from the skin. Losses of non-collagenous nitrogen and wet weight from muscle, carcass and viscera were similar and comparable to those in the whole body; from the skin they were small, possibly owing to the fact that hair is in a stable condition and can be lost only by falling out or friction. There was no loss of weight of non-collagenous N from the femora.

DISCUSSION

Although the quantitative distribution of collagen within the bodies of some animals can be approximately predicted from the literature, our results for mice appear to be the first direct measurements, except for a single guinea-pig analysed by Lightfoot & Coolidge (1948), who used a method of doubtful specificity (Harkness, 1952). Our results clearly indicate the importance of collagen among the body proteins, of which it constitutes about one-sixth in control animals. Of total body collagen, no less than 40% is in the skin, which forms only some 17% of the weight of the body. The latter figure agrees with that of 17·6% given by Welcher & Brandt (1903) for the mouse. The mouse, however, is a small animal and one might expect the proportion of the total collagen content of the body in the skin to be less in larger animals, which have a smaller relative skin surface area. It appears in fact that the thickness of the skin increases in proportion to other linear dimensions, since it forms about the same proportion of body weight in large as in small animals. Some figures from the literature of the percentage of the body weight contributed by the skin are 17% in the rat (Jackson & Lowry, 1912) 14·7% in the cat (Seldmair, 1899), 7·1–12·5% in sheep (Hennenberg, 1881), 20% including subcutaneous fatty tissue which forms half to three quarters or more of the total in the human new born and adult (Vierordt, 1906), 7–10% in cattle (Lawes & Gilbert, 1859; Moulton, Trowbridge & Haigh, 1922; Edinger, 1925), 15·5% in an elephant (Quiring, 1939).

Nitrogen constituted 3·6% of the wet weight in skin from control animals, and of this 28% was collagenous nitrogen. Eisele & Eichelberger (1945) found 3·37% nitrogen in wet, fat-free, human skin, of which no less than 74% was collagenous. The smaller representation of collagenous nitrogen in our animals was presumably due in part to the fact that hair had not been removed.

The rate of loss of collagen over the whole experimental period on the protein-free diet was slow compared with some of the other rates of loss recorded in the literature (see Harkness & Moralee, 1956). Our experiments agree with those of Neuberger *et al.* (1951) and Neuberger & Slack (1953) in showing collagen to have greater metabolic stability than other proteins taken as a whole. Neuberger & Slack (1953), however, found higher rates of incorporation of ^{14}C glycine into the collagen of bone and liver of adult rats than into skin, whereas in our experiments skin showed greater change than bone. Our experiments provided no evidence of change in bone, although this tissue is generally regarded as having relatively high metabolic activity. The control of the collagen content of bone is, however, peculiar and this may well explain our results. Reabsorption of calcium salts from bone appears to involve dissolution of whole bone tissue including the organic matter and it seems generally agreed that in the reabsorption of bone which accompanies normal growth the organic component, which is principally collagen and the inorganic components are removed almost simultaneously (e.g. Kölliker, 1873; McLean & Bloom, 1940). When calcium salts are stored in or released from the skeleton in response to variation in intake of calcium in the diet, both components appear to change together (Bauer, Aub & Allbright, 1929; Bell, Cuthbertson & Orr, 1941; Walker & Arvidsson, 1954). Both components are also involved in the variations in calcium storage which occur in connexion with pregnancy and lactation (Ellinger, Duckworth, Dalgarno & Quenouille, 1952), and egg-laying (Bloom, Bloom & McLean, 1941). Bone reabsorption resulting from the administration of parathyroid extracts also seems to involve both components (Bauer *et al.* 1929; McLean & Bloom, 1941). The most potent cause of loss of material from bone appears to be immobilization and disuse. In this case also salts and organic matrix are lost together (Allison & Brooks, 1921).

There is no evidence to show why skin collagen should respond to protein depletion more readily than collagen elsewhere in the body. Little is known of the mechanisms controlling skin size, but it has been suggested (Billingham & Medawar, 1955; Abercrombie & James, 1957) that skin may grow in response to tension. The converse may be true: that a reduction in tension, as when weight is rapidly lost, may lead to regression in skin area and weight.

We are grateful to the Nuffield Foundation for a grant towards this work, and to Miss Shirley M. Fitch and Miss Sylvia Jenkins for skilled technical assistance.

SUMMARY

1. The effect of a protein-free diet on the collagen content of the body has been investigated in adult mice.

2. Collagen was found to form 2–3 % of the wet weight of normal mice. Collagenous nitrogen formed 15–20 % of the total nitrogen.

3. The experimental diet was given for 17–20 days and was found to produce a significant loss of collagen from the body, though this (10 %) was smaller than the loss of body weight (45 %) or non-collagenous nitrogen (35 %).

4. Investigation of individual parts of the body (quadriceps femoris muscle, femur, viscera, skin and carcass) showed that the greater part of the loss of collagen (60 %) was from the skin, which also lost a higher proportion (16 %) of its original collagen than any other part.

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THE ACTION OF SYMPATHOMIMETIC AMINES IN ANIMALS TREATED WITH RESERPINE

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The impression left in the minds of those who have read the account of the sympathomimetic amines written by Barger & Dale in 1910 has been that these substances in the main were found to differ only quantitatively in activity. From time to time, however, there have been observations which furnished a hint of qualitative differences. Perhaps the first of these was the discovery by Tainter & Chang (1927) that under the influence of cocaine the action of tyramine was abolished, although, as Fröhlich & Loewi had shown in 1910, the action of adrenaline was potentiated. In 1931 Burn & Tainter observed that tyramine and ephedrine had no action on the denervated pupil, though to adrenaline it was supersensitive. In 1932, Burn recorded that denervation of the cat's foreleg by removal of the stellate ganglion led to a loss of the vasoconstrictor action of tyramine and of ephedrine, though not of that of adrenaline. Burn also found that in the perfused dog's hind leg tyramine and ephedrine had, compared with adrenaline, only a fraction of the constrictor action they would be expected to have, judging from the pressor effect in the spinal cat. He observed, however, that the constrictor action could be restored if adrenaline was added to the blood perfusing the hind leg, and that this restoration could not be effected by adding pituitary (posterior lobe) extract instead.

Fleckenstein & Burn (1953) found that after denervation of the nictitating membrane it became more sensitive only to those amines which were catechol derivatives; that to the derivatives of phenylethanolamine it was less sensitive and to the derivatives of phenylethylamine it was nearly insensitive. Fleckenstein & Bass (1953) and Fleckenstein & Stöckle (1955) showed that the normally innervated nictitating membrane after full doses of cocaine behaved in exactly the same way.

These various observations have remained incoordinated and unexplained. Recently, however, Carlsson, Rosengren, Bertler & Nilsson (1957) found that, in cats treated with reserpine, the intravenous injection of tyramine had no pressor effect. In explanation they said that 'tyramine belongs to a group of sympathomimetic amines which are fully active only in the presence of an intact adrenergic system'. We have recently observed (Burn & Rand, 1957, 1958*b*) that when rabbits and dogs were treated with reserpine, the noradrenaline-like substance demonstrated by Schmiterlöw (1948) to be present in the wall of the aorta disappeared. We also observed that the constrictor action of nicotine in the perfused rabbit's ear disappeared, and were led to the conclusion that nicotine exerted this constrictor action by releasing a noradrenaline-like substance from the vessel wall or perhaps from chromaffin cells in its neighbourhood.

It therefore seemed to us possible that the failure of tyramine to exert its pressor action in the cat after treatment with reserpine was due to the fact that tyramine normally acted by releasing a noradrenaline-like substance from the vessel walls. We now describe experiments to test the action of a series of sympathomimetic amines from this point of view.

METHODS

Observations of four kinds have been made. The effect of sympathomimetic amines has been examined (1) on the blood pressure, the spleen and the nictitating membrane of the spinal cat; (2) on the arterial resistance and venous outflow of the perfused dog hind leg; (3) on the outflow of the perfused rabbit ear; and (4) on spiral strips of rabbit aorta set up in an isolated organ bath.

Spinal cats were prepared under ether anaesthesia, dividing the cord at the 2nd cervical vertebra and destroying the brain. Records of the spleen volume and of the contractions of the nictitating membrane were made as described by Burn (1952). For the hind leg perfusion one dog was bled under ether anaesthesia from the carotid artery into a jar containing heparin, and its lungs were prepared for perfusion by one of a pair of Dale–Schuster (1928) pumps. Perfusion was begun through the lungs, which were ventilated by oxygen with 5% CO_2. A second dog was then anaesthetized. After dividing the rectum and the inferior mesenteric artery between ligatures, the right external iliac artery was tied, and a cannula was inserted pointing towards the aorta. The body wall was divided in sections between ligatures along the course of the left external iliac artery. Poupart's ligament was cut. Branches of the left external iliac from the aorta to about 1 cm below Poupart's ligament were tied, as were also the corresponding veins. The continuation of the aorta below the origin of the external iliac arteries was tied. When the dissection was complete, perfusion was begun by the second pump through the left hind leg at the moment of tying the aorta to arrest the natural circulation.

In some experiments the left lumbar sympathetic chain was prepared for stimulation; in these the dog was first eviscerated and the left kidney removed. After the arrest of the natural circulation, the dog was cut in half, the perfusing cannula was tied into the aorta and the blood collected from the vena cava. By this method the ganglia of the sympathetic chain were perfused. In these experiments there was an interval of 7 min between the arrest of the natural circulation and the start of the perfusion. In all perfusions mass ligatures were tied so as to enclose the whole circumference of the body wall above the area perfused. An outflow record was obtained by using Stephenson's recorder (1949). When stimulating the sympathetic chain shielded electrodes, each a silver plate 3 mm wide, were used; stimuli were square-wave pulses of 2·5 mA strength, 1 msec duration and 20–25/sec frequency.

The perfusion of the rabbit ear was arranged as previously described (Burn, 1952) using Stephenson's outflow recorder (1948).

Reserpine was dissolved in 20% ascorbic acid solution making the solution up to 10 mg/ml. as required. Injections were made intraperitoneally. Dogs were given 0·5 mg/kg on two successive days, the dog being killed on the third day. Cats were given 2·5–5·0 mg/kg on two successive days, and rabbits were given 3 mg/kg on two successive days.

Fig. 1. Upper record is that of the nictitating membrane; lower record that of blood pressure in the spinal preparation. (*a*) Normal cat; injection of 10 μg noradrenaline produced a small rise of blood pressure; 4 mg tyramine hydrochloride produced a large rise of blood pressure and large contraction of the nictitating membrane. (*b*) Cat treated with reserpine. Note that the effects were reversed; 4 mg tyramine had a very slight effect on the blood pressure and none on the nictitating membrane; 10 μg noradrenaline had a large effect on the blood pressure. (*c*) Same as (*b*); after the intravenous infusion of 0·12 mg noradrenaline, the effect of 4 mg tyramine on the blood pressure was much greater than in (*b*).

RESULTS

Action of tyramine

Fig. 1 shows records taken from experiments in two spinal cats, the one a normal cat, and the second a cat treated with reserpine. In Fig. 1*a* the injection of 10 μg noradrenaline into the normal cat caused a small rise of blood pressure, whereas the injection of 4 mg tyramine hydrochloride caused a large rise of blood pressure and a large contraction of the nictitating membrane. In Fig. 1*b* the same injections were made into a cat treated with reserpine. The effect of 4 mg tyramine hydrochloride was very small and there was no contraction of the nictitating membrane, while the effect of 10 μg noradrenaline was very much larger than in the normal cat. A solution of noradrenaline was then prepared in a burette and the burette was connected to the femoral vein. An infusion of noradrenaline at a uniform rate was begun and maintained until 0·12 mg noradrenaline had entered the cat. The infusion caused a large rise of blood pressure, but although the rate of infusion was kept constant, the blood pressure fell as the infusion continued, though the extent of this fall varied in different experiments. At the end of the infusion the point at which the blood pressure returned to its former level was noted, and an injection of 4 mg tyramine was made again, as shown in Fig. 1*c*.

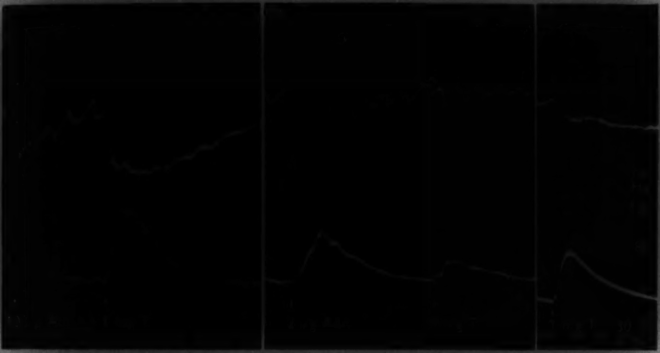

Fig. 2. Upper record is that of the spleen volume; lower record that of blood pressure, in the spinal preparation. (*a*) Normal cat. (*b*) Cat, treated with reserpine, in which 2 μg adrenaline had a greater effect on the spleen volume than 10 μg in (*a*), and in which 1 mg tyramine had no action, though it contracted the spleen in (*a*). An infusion of noradrenaline was then made, after which (*c*) 1 mg tyramine again caused contraction.

The rise of blood pressure was much greater than in Fig. 1*b* and there was also a small contraction of the nictitating membrane which was absent in Fig. 1*b*. The events shown in Fig. 1 were observed in many experiments. When the pressor action of tyramine had been restored, as in Fig. 1*c*, then repeated injections of tyramine had a declining effect, but a further infusion of noradrenaline would restore it a second time.

In other experiments the spleen volume was recorded, as shown in Fig. 2. In a normal cat (Fig. 2*a*), the injection of 10 μg adrenaline and of 1 mg tyramine produced a contraction of the spleen volume. In a cat treated with reserpine (Fig. 2*b*), 2 μg adrenaline produced a greater contraction of the spleen volume than 10 μg in the normal cat, and 1 mg tyramine had no appreciable action on the spleen volume. After the intravenous infusion of noradrenaline, both the pressor action of 1 mg tyramine and its action on the spleen volume were partly restored (Fig. 2*c*). Observations were also made by perfusing the hind leg of the dog. In a preparation from a dog previously treated with reserpine the injection of 0·25 mg tyramine had no constrictor effect as Fig. 3*a* shows. Stimulation of the sympathetic chain after the injection of 0·1 mg atropine to exclude the cholinergic fibres (Bülbring & Burn, 1935) also had no effect. After the slow addition of 0·41 mg noradrenaline to the reservoir and after sufficient time had elapsed for the effect of this noradrenaline on the arterial resistance to have passed off, stimulation was then effective, as shown in Fig. 3*b*, and the injection of 0·25 mg tyramine caused a rise in mean arterial resistance of 56 mm.

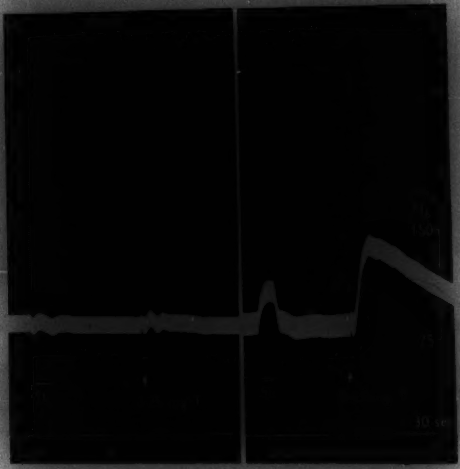

Fig. 3. Records from perfusion of hind leg of dog treated with reserpine. Upper record is venous outflow; lower record is pressure in the arterial cannula. In (*a*) at St., stimulation was applied to the lumbar sympathetic after injecting 0·1 mg atropine to exclude cholinergic fibres; it had no effect. Similarly, injection of 0·25 mg tyramine into the cannula had no effect. (*b*) The records were taken after the addition of 0·41 mg noradrenaline to the venous reservoir during 20 min. When the pressure returned to its previous level, stimulation at St. caused arterial constriction and a fall in outflow. The injection of 0·25 mg tyramine caused vasoconstriction and a fall in outflow.

Since reserpine is known to displace hydroxytryptamine from sites where it is bound, and since Woolley & Shaw (1953) state that hydroxytryptamine is present in arteries, it was possible that the failure of tyramine to exert a pressor effect in the reserpine-treated cat was due to absence of hydroxytryptamine. We therefore carried out experiments to see if an infusion of hydroxytryptamine would restore the pressor effect of tyramine, but we failed to observe any sign of such an action either in the spinal cat or in the perfused hind leg of the dog; we were surprised to find that the infusion of amounts from 1 to 2 mg hydroxytryptamine had little or no effect on the blood pressure.

Phenylethylamine

We obtained results with phenylethylamine very similar to those obtained with tyramine, and an illustration in Fig. 4 shows the changes in the nictitating membrane as well as those in the blood pressure when phenylethylamine was examined. The observations in Fig. 4 were made in a cat treated with reserpine. In Fig. 4*a* the injection of 4 mg phenylethylamine had little effect on the blood pressure or on the nictitating membrane, while the injection of 10 μg noradrenaline had a large effect on both. An infusion of a total of 1 mg noradrenaline was then given, and when its effect on the blood pressure had passed off, the injection of 10 μg noradrenaline now had a much smaller

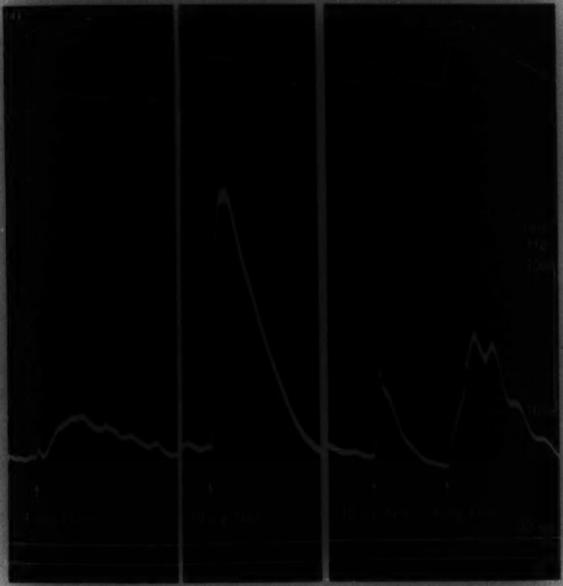

Fig. 4. Records as in Fig. 1 from spinal cat treated with reserpine. (*a*) Injection of 4 mg phenylethylamine had a small effect; (*b*) 10 μg noradrenaline had a large effect on blood pressure and also on the nictitating membrane; (*c*) after the intravenous infusion of noradrenaline during 20 min the injections were repeated. The effect of 10 μg noradrenaline was much smaller on the blood pressure and on the nictitating membrane than in (*b*), the effect of 4 mg phenylethylamine was greater on the blood pressure and much greater on the nictitating membrane than in (*a*).

effect in both records, while the injection of 4 mg phenylethylamine had a much greater effect, the increased contraction of the nictitating membrane being particularly clear. Observations were also made with phenylethylamine in the perfused hind leg of the dog. An example of these is given in Fig. 5, taken from an experiment on a dog given reserpine beforehand. In Fig. 5*a* the injection of 1 mg phenylethylamine caused a small increase in arterial resistance attended by a slight rise and fall in the venous outflow. Between Fig. 5*a* and Fig. 5*b*, an infusion of 1 mg hydroxytryptamine was made; when the injection of phenylethylamine was repeated, apart from a very slight increase in outflow, there was no effect at all. An infusion of 1 mg noradrenaline was then given, and when its effect on the vascular tone had passed off, the injection of phenylethylamine caused a large vasoconstriction and decrease in outflow (Fig. 5*c*.)

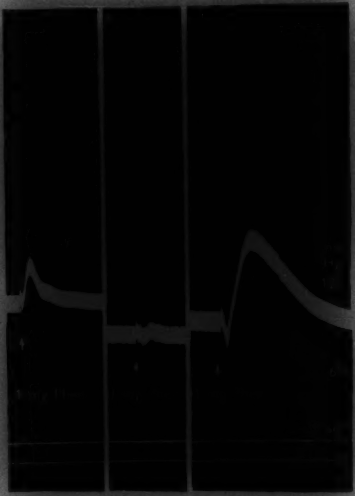

Fig. 5. Records of perfusion of hind leg of dog treated with reserpine. Upper record is venous outflow; lower record is pressure in arterial cannula. (*a*) Injection of 1 mg phenylethylamine produced small constriction and small increase in outflow followed by a decrease; (*b*) after the infusion of 1 mg hydroxytryptamine the injection of phenylethylamine had less effect than before; (*c*) after the infusion of 1 mg noradrenaline, the injection of 1 mg phenylethylamine had a much greater constrictor action.

The catecholamines

From the evidence so far given and illustrated in Figs. 1 and 4, the effect of reserpine was to remove the pressor action of tyramine and of phenylethylamine and to increase that of noradrenaline. The increased action of noradrenaline was, however, depressed by an infusion of noradrenaline. Noradrenaline is a derivative of catechol, and we tested other catecholamines to see whether their pressor action in the reserpine-treated cat was depressed by an infusion of noradrenaline. The results in one experiment are shown in Table 1, in which the substance neosynephrine, which has the same structure as adrenaline except that it lacks the —OH group in the 4 position in the ring, was also tested. All the substances in Table 1 were active pressor agents in the reserpine-treated cat in contrast to tyramine and phenylethylamine, and all were made less active by an infusion of noradrenaline.

Since the constrictor action of tyramine in the perfused hind leg was increased not only by the addition of noradrenaline to the blood but also by the addition of adrenaline as shown by Burn (1932*a*), we decided to test the effect of infusing dopamine. Fig. 6*a* shows the effect of injecting 4 mg tyramine in a cat treated with reserpine. The injection raised the blood pressure by 22 mm. A total of 1 mg dopamine was then infused. When the effect of this infusion on the blood pressure had subsided, the injection of 4 mg tyramine then raised the blood pressure by 46 mm, but did not cause contraction of the nictitating membrane. A further infusion of 4 mg dopamine was given. Afterwards the injection shown in Fig. 6*b* was made, which caused no greater rise of pressure than in 6*a*, but caused contraction of the nictitating membrane. Finally, an infusion of 1 mg noradrenaline was given, and after it injection of 4 mg tyramine caused the large effects shown in Fig. 6*c*.

TABLE 1. Effect of infusing noradrenaline on pressor action in cat treated with reserpine (spinal preparation)

Substance	Dose (μg)	Pressor action (mm Hg) Initial	Pressor action (mm Hg) After noradrenaline infusion
Noradrenaline	3	104	26
Adrenaline	20	144	54
Epinine	100	132	58
Dopamine	200	122	34
Neosynephrine	100	150	38

The phenylethanolamines

These results established the contrast between substances with an ethylamine side chain which were not catecholamines on the one hand, and substances which were in the main catecholamines on the other. We were fortunate in being able to examine phenylethanolamine in the form of its two optical isomers which were prepared by Pratesi & Grassi (1953). The one form, the (−)-form has the same configuration as (−)-adrenaline, while the (+)-form has the same configuration as (+)-adrenaline. We found that while the (+)-form behaved very much as tyramine or phenylethylamine the (−)-form resembled the catecholamines much more, though not completely.

Fig. 7 shows the action of the two forms in a normal cat and also in a cat treated with reserpine. In Fig. 7*a* the pressor action of 4 mg (+)-phenylethanolamine was similar to that of tyramine, the pressure not rising steeply and the rise being prolonged. The pressor action of 2 mg (−)-phenylethanolamine was slightly greater, but the pressure rose more steeply and fell more rapidly. In Fig. 7*b* the pressor action of 4 mg (+)-phenylethanolamine in the reserpine-treated cat had almost disappeared, as also had the action on the nictitating membrane; the pressor action of 2 mg (−)-phenylethanolamine was somewhat smaller than in the normal cat but it was even more adrenaline-like in shape and there was also a contraction of the nictitating membrane.

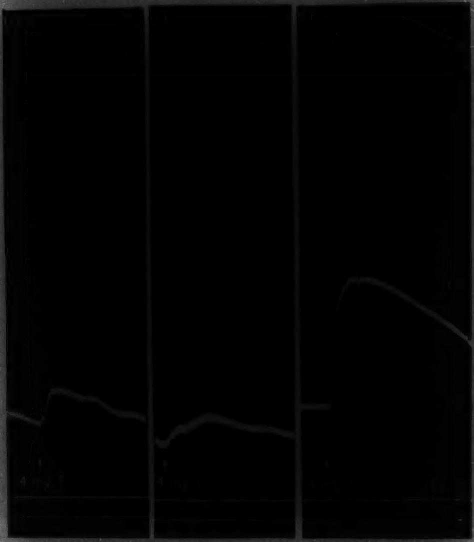

Fig. 6. Records as in Fig. 1 from spinal preparation of cat treated with reserpine. (*a*) Injection of 4 mg tyramine. (*b*) Repetition of injection of 4 mg tyramine after the infusion of 5 mg dopamine; the injection produced a contraction of the nictitating membrane. (*c*) Repetition of injection of 4 mg tyramine after the infusion of 1 mg noradrenaline; the injection caused a much greater rise of blood pressure and contraction of the nictitating membrane.

These results suggested that the action of (+)-phenylethanolamine was like that of tyramine or phenylethylamine. This conclusion was supported by observations in which noradrenaline was infused into the spinal cat; after the effect of noradrenaline on the blood pressure had passed off, the pressor effect of (+)-phenylethanolamine was increased. Thus in one experiment (see Fig. 8) 4 mg (+)-phenylethanolamine caused a rise of 24 mm with no contraction of the nictitating membrane, but after the infusion of noradrenaline the same amount caused a rise of 72 mm with a contraction of the nictitating membrane of 15 mm.

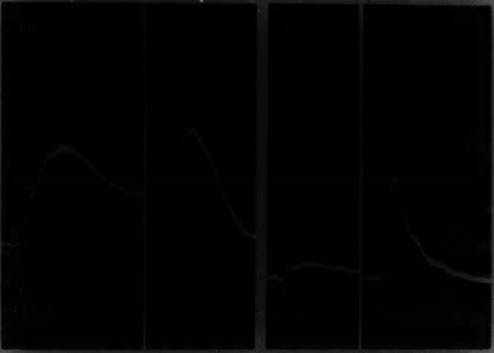

Fig. 7. Records as in Fig. 1. (*a*) From spinal preparation of normal cat; injection of 4 mg (+)-phenylethanolamine and of 2 mg (−)-phenylethanolamine; the rise in blood pressure caused by the second injection was more 'adrenaline-like' and less 'tyramine-like' than that of the first injection. (*b*) From cat treated with reserpine; the injection of 4 mg (+)-phenylethanolamine had almost no effect on the blood pressure and none on the nictitating membrane; the injection of 2 mg (−)-phenylethanolamine was still more 'adrenaline-like', and there was a small contraction of the nictitating membrane.

TABLE 2. Action of 2 mg (−)-phenylethanolamine in cats treated with reserpine

	Before infusion of noradrenaline		After infusion of noradrenaline	
Cat	Pressor effect (mm)	Nict. memb. contraction (mm)	Pressor effect (mm)	Nict. memb. contraction (mm)
1	70	18	4	12
2	164	26	124	39
3	68	19	100	25
4	31	10	40	7
Mean	83	18	67	21

The action of (−)-phenylethanolamine in the reserpine-treated cat was difficult to define. The results of four experiments are shown in Table 2, which shows that the mean responses were very similar before and after the infusion of noradrenaline, so that this substance did not fall into the class of catechol amines on the one hand, or into the class which included tyramine, phenylethylamine and (+)-phenylethanolamine on the other. That it had some affinity to this class was shown by an experiment in a perfused dog's hind leg, in which the injection of 0·5 mg (−)-phenylethanolamine increased the arterial resistance by 17 mm, but after the infusion of noradrenaline the injection increased it by 46 mm.

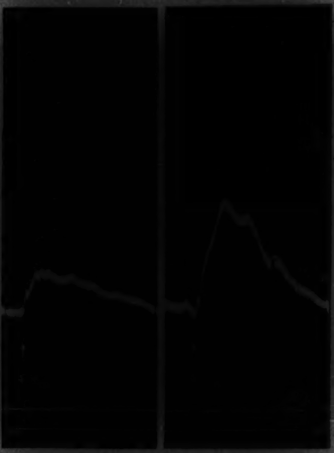

Fig. 8. Records as in Fig. 1 from spinal preparation of cat treated with reserpine. Injection of 4 mg (+)-phenylethanolamine (*a*) before and (*b*) after the intravenous infusion of 1 mg noradrenaline; in (*b*) the injection caused a larger rise of blood pressure and a contraction of the nictitating membrane.

The action of ephedrine

Ephedrine is a derivative of phenylethanolamine in which a methyl group is attached to the α carbon (adjoining the amine group) and a methyl group is also attached to the nitrogen, as in adrenaline. Ephedrine has a much longer duration of action than the substances considered hitherto. Its action on the nictitating membrane is specially prolonged. There is general agreement that this greatly increased duration of action is due to the presence of the methyl group on the α carbon atom, which prevents the destruction of ephedrine by amine oxidase. The form of ephedrine used was the (−)-isomer.

Fig. 9 shows the effect of 2 mg ephedrine on the blood pressure and nictitating membrane of (*a*) a normal cat, and (*b*) a cat treated with reserpine. In each the injection of ephedrine was the first injection after making the spinal preparation. In normal cats successive injections caused decreasing effects, but the decrease was much more rapid in cats treated with reserpine. It was not so easy to restore the pressor action of ephredine in the reserpine-treated cat by an infusion of noradrenaline as to restore the pressor action of the substances already described. Fig. 10, however, shows a preparation in which an injection of no more than 10 μg noradrenaline had some restoring action, for 1 mg ephedrine, given after it, had more pressor action than when given before, and also more action on the nictitating membrane. In the record it is seen that noradrenaline caused contraction of the nictitating membrane; this it had failed to do when the same injection was made on three previous occasions in this experiment. We concluded that the contraction was a consequence of the injection of ephedrine and was due to inhibition of the amine oxidase in the nictitating membrane.

Fig. 9. Records from spinal preparation as in Fig. 1. (*a*) Normal cat; injection of 2 mg ephedrine. (*b*) Cat treated with reserpine; the injection of 2 mg ephedrine had much less effect.

In the perfused hind leg of the dog treated with reserpine, the small constrictor effect of ephedrine was increased by an infusion of noradrenaline, though not greatly. We made repeated attempts to see if the pressor action of ephedrine was restored by an infusion of hydroxytryptamine. All these experiments failed, and indeed we found that hydroxytryptamine reduced the pressor action. For example, in an experiment on a normal cat the injection of 2 mg ephedrine at approximately 30 min intervals caused rises of 126, 66, 42, 42 mm. Then five injections each of 0·2 mg hydroxytryptamine were given, after which the injection of 2 mg ephedrine caused a rise of only 3 mm.

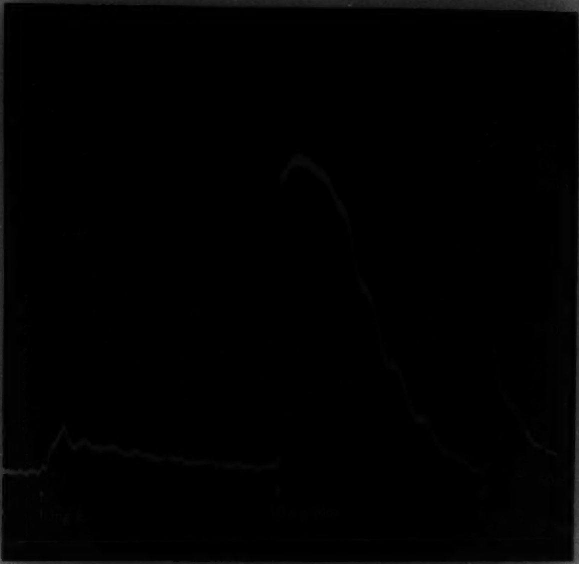

Fig. 10. Records as in Fig. 1 from spinal preparation of cat treated with reserpine. Injection of 1 mg ephedrine at E. had very little pressor action; injection of 10 μg noradrenaline caused large rise of blood pressure and a contraction of the nictitating membrane, which had not been seen previously when 10 μg noradrenaline was injected. The contraction may have been due to inhibition of the amine oxidase by the ephedrine which had been injected just before. An injection of 1 mg ephedrine immediately after the response to noradrenaline produced a greater pressor action than before and a contraction of the nictitating membrane.

Action of amphetamine

The action of amphetamine was very like that of ephedrine. In the reserpine-treated cat the rise of blood pressure was small, and there was no contraction of the nictitating membrane. We were able to demonstrate the restoration of the effect on the nictitating membrane by an infusion of noradrenaline, as illustrated in Fig. 11 in which, before the infusion of noradrenaline, we tested the preparation by injecting tyramine to see that the injection of reserpine had been effective. We also increased the constrictor action in the perfused hind leg of the dog by an infusion of noradrenaline, but not greatly.

Effect of sympathetic stimulation

We observed in several experiments that in cats treated with reserpine, in which tyramine, phenylethylamine and other amines acting like them had

Fig. 11. Records as in Fig. 1 from spinal preparation of cat treated with reserpine. (*a*) Injection of 4 mg tyramine to demonstrate that the animal had been effectively treated with reserpine, in which case the injection of 2 mg amphetamine would have had little effect. (*b*) Injection of 2 mg amphetamine after intravenous infusion of 1 mg noradrenaline; the amphetamine produced a large effect.

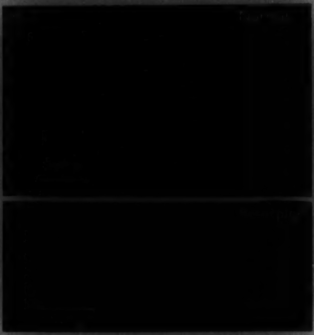

Fig. 12. (*a*) Contraction of nictitating membrane of normal cat in response to 10 sec maximal stimulation of cervical sympathetic chain. (*b*) Response to the same stimulation in a cat treated with reserpine in which the injection of 4 mg tyramine had no effect on the membrane.

little effect on the blood pressure and no effect on the nictitating membrane, maximal stimulation of the cervical sympathetic chain still caused contraction of the nictitating membrane, as shown in Fig. 12*b*. The contraction followed the stimulus after a slightly greater delay and both the rate of rise and rate of fall were slower than in the normal cat (Fig. 12*a*). This persistence of the effect of nerve stimulation was observed by Muscholl & Vogt (1958).

Action of various amines in the perfused rabbit ear

In a previous paper (Burn & Rand, 1958*b*) we showed that the vasoconstrictor action of nicotine in the perfused rabbit ear was absent in rabbits treated with reserpine. We therefore investigated the constrictor action of tyramine, ephedrine and amphetamine in the perfused rabbit ear, a record being obtained of the outflow, so that the degree of constriction could be measured on the drum. Observations were made in ears from normal rabbits and also in ears from rabbits previously treated with reserpine, and they are summarized in Table 3. The constrictor effect of all three substances was reduced in the ears of rabbits treated with reserpine. The effect of the amines was not abolished, however, and this was in contrast to the effect of nicotine, which was regularly abolished. The same difference was observed in spiral strips of rabbit aorta by our colleague Dr Damrong Bejrablaya. Strips taken from rabbits treated with reserpine were not stimulated by nicotine, but were stimulated by tyramine and by amphetamine.

TABLE 3. Mean constrictor action in perfused rabbit ear

Substance	Dose (μg)	Normal (mm)	Reserpine-treated (mm)
Tyramine	2	28 (4)	0 (1)
	4	53 (8)	15 (4)
	8	92 (3)	21 (8)
	16	—	41 (1)
Ephedrine	4	18 (1)	
	8	47 (5)	17 (3)
	16	49 (2)	22 (6)
	32	—	56 (2)
Amphetamine	1	45 (6)	3 (3)
	2	43 (4)	3 (4)
	4	49 (3)	11 (4)

The figures in parentheses are the numbers of observations.

A further important difference between the action of the amines and that of nicotine was that the action of the amines in the perfused rabbit ear was not affected by hexamethonium (see Fig. 13) whereas the action of nicotine was always abolished.

Fig. 13. Record of outflow from perfused rabbit ear. (*a*) Diminution of outflow after injection of 5 μg nicotine acid tartrate. (*b*) Diminution of outflow after injection of 8 μg tyramine hydrochloride; outflow unaffected by 5 μg nicotine following 0·1 mg hexamethonium (H.), but outflow decreased as before by 8 μg tyramine following 0·1 mg hexamethonium (H.).

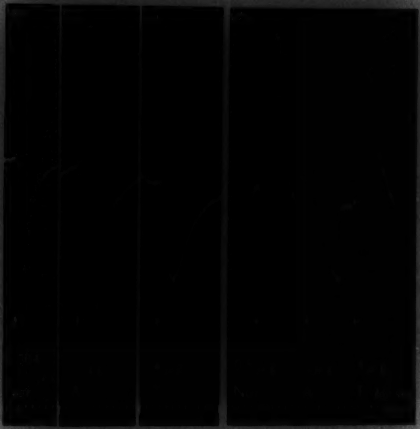

Fig. 14. Record of outflow from perfused rabbit ear. Diminution of outflow caused by (*a*) 0·004 μg noradrenaline, (*b*) 2 μg amphetamine, (*c*) 4 μg tyramine. Between (*c*) and (*d*) tolazoline (Priscol) 0·2 mg/ml. was added to the perfusing fluid; (*d*) shows that 0·5 μg noradrenaline caused an increase of outflow, 2 μg amphetamine caused a decrease of outflow and 4 μg tyramine caused an increase of outflow.

Reversal of effects of amines by tolazoline

Knowing that the effect of noradrenaline is reversed by tolazoline (Priscol) in the perfused ear, becoming dilator (Burn & Hutcheon, 1949), we tested the effect of adding tolazoline to the perfusing fluid. We found, as shown in Fig. 14, that when the effect of noradrenaline was reversed, the effect of tyramine was also reversed, but, except in one experiment, the effect of amphetamine was not reversed. Similarly, we found that the effect of ephedrine was reversed, but not that of phenylethylamine. The results are shown in Table 4.

TABLE 4. Perfusion of rabbit ears with tolazoline (20 μg/ml.); five experiments with each drug

	Constrictor action reversed (no. of experiments)
Tyramine	5
Phenylethylamine	0
Amphetamine	1
Ephedrine	5

Fig. 15. Record of outflow from perfused rabbit ears, to show the effect of cocaine. (*a*) shows fall in outflow due to 4 μg tyramine, (*b*) shows fall in outflow due to 0·004 μg noradrenaline, 8 μg ephedrine, 1 μg amphetamine and 3·5 μg nicotine acid tartrate. Between (*b*) and (*c*) cocaine 5×10^{-4} g/ml. was added to the perfusing fluid; in (*c*) the effect of tyramine was reversed to dilatation, that of noradrenaline was unchanged, those of ephedrine, amphetamine and nicotine were abolished.

Abolition of effects of amines by cocaine

Tainter & Chang (1927) showed that the pressor action of tyramine was reduced or abolished by cocaine. We therefore tested the effect of adding cocaine to the fluid perfusing the ears of normal rabbits. We observed that the constrictor action of tyramine, ephedrine, amphetamine and nicotine was abolished at a time when that of noradrenaline was unaffected. Such a result is shown in Fig. 15; in addition, the effect of tyramine was reversed by cocaine to a dilator action. This change in the spinal cat was described previously by Burn & Tainter (1931).

DISCUSSION

The results described in this paper suggest that the sympathomimetic amines may be divided into two classes, one consisting of substances like noradrenaline and adrenaline, which act on vessels in both normal and reserpine-treated animals, and the other consisting of substances like tyramine which act only on vessels of normal animals and have little or no action on those treated with reserpine. We have previously demonstrated that reserpine treatment has three consequences; it causes the store of noradrenaline-like substance which is present in the walls of the rabbit aorta to disappear; it causes the noradrenaline-like substance in the skin of the rabbit ear to disappear; and it causes the chromaffin cells adjoining the vessels in the rabbit ear to disappear (Burn & Rand, 1958*b*). These observations suggest that substances like tyramine, which lose their pressor and constrictor action in the reserpine-treated animal, act in the normal animal by releasing a noradrenaline-like substance which, when released, exerts the pressor and constrictor action. Our observations do not indicate whether this is a general release into the blood stream or whether it is only a local release. This suggestion receives support from the observation that the constrictor action of tyramine can be restored in the vessels of the reserpine-treated animals by perfusing noradrenaline through the vessels for a short period. Both during the infusion and also after it, when the constrictor action of this noradrenaline has passed off, the injection of tyramine, or of substances which act like it, once more causes constriction. One of us showed (Burn, 1932*a*) that a similar effect was obtained by perfusing adrenaline through the vessels.

The vessels of the reserpine-treated animals differ from those of the normal animal, not only in being insensitive to tyramine and substances acting like it, but also in being highly sensitive to the action of noradrenaline and adrenaline. Thus 0·1 μg noradrenaline was seen to have as great a constrictor effect in the perfused hind leg of a reserpine-treated dog as 2–4 μg noradrenaline in that of a normal dog. When noradrenaline was infused to restore the sensitivity to tyramine, the high sensitivity to noradrenaline was reduced to its normal level.

These observations indicate that there is a store of material in or near the vessel walls from which tyramine, ephedrine, amphetamine and other similar substances release either noradrenaline or adrenaline or both. The action of these 'adrenaline-releasers' differs from that of nicotine and of acetylcholine since it is not abolished by hexamethonium. It is thus conceivable that while nicotine and acetylcholine act on the chromaffin cells which Adams-Ray & Nordenstam (1956) have observed in human skin and which Mr E. H. Leach found in the skin of the rabbit ear (Burn & Rand, 1958*b*), liberating adrenaline from these cells, tyramine, ephedrine and similar amines act by displacing what may be mainly noradrenaline from the vessel wall. The fact of importance, however, is that the store does not appear to be an inert store, for its presence reduces the response of the vessel to an injection of adrenaline or noradrenaline into the blood stream. From this it appears that there may normally be some discharge from the store, the noradrenaline-like substance which is set free occupying some of the receptors and playing a part in maintaining the tone. If this were not so, it is difficult to understand how the sensitivity of the vessel to injected noradrenaline could be diminished by the presence of the store. We have already demonstrated such a discharge from the store in the heart by its effect on the rate of the pace-maker (Burn & Rand, 1958*a*). Thus we appear to have in this store a new factor in the control of vascular tone.

Our observations have thrown some light on the action of cocaine. The vessels of a cat treated with reserpine react in a similar manner to those of a normal cat which has received an injection of cocaine. In both there is supersensitivity to the action of noradrenaline and adrenaline, and in both there is greatly depressed sensitivity to the action of tyramine and ephedrine. This similarity suggests that the action of cocaine may be to arrest the release of the noradrenaline-like substance from the store. In the presence of cocaine tyramine and ephedrine are no longer able to act as 'adrenaline-releasers', and in the absence of a release the vessel at once becomes much more sensitive to the action of injected noradrenaline or adrenaline. We may note that cocaine also prevents the release by nicotine.

One of us has expressed the opinion (Burn, 1956) that the potentiation of adrenaline by cocaine is explained by inhibition of amine oxidase; the present evidence indicates that this opinion is wrong, as others (Bacq, 1949) have contended. It may, however, be wise to keep in mind the possibility that, in the action of cocaine, diminished destruction of adrenaline plays a part.

There is also a similarity between tissues denervated by degeneration of their sympathetic supply and the tissues of an animal treated with reserpine. After denervation the tissues become supersensitive to adrenaline and noradrenaline, and less sensitive to the action of tyramine and ephedrine. It is interesting to note that Fleckenstein & Bass (1953) and Fleckenstein & Stöckle (1955) have demonstrated a close similarity in the behaviour of the nictitating membrane after denervation to its behaviour after treatment with cocaine. Towards the catechol amines it shows supersensitivity in both cases; towards the phenylethanolamines it shows diminished sensitivity in both cases; towards the phenylethylamines it shows greatly diminished sensitivity in both cases. We know from the work of von Euler & Purkhold (1951) that after degeneration of sympathetic fibres there is a loss mainly of noradrenaline from the spleen, the kidney, the liver and the salivary glands. We know from the work of Goodall (1951) of a similar loss in the heart. Examination of the effect of denervation of the vessels, which has not yet been made, may show a similar loss, and if so it may indicate that the changes in the reaction of vessels caused by denervation are to be explained in the same way as those caused by injections of reserpine.

The evidence that an infusion of noradrenaline, either intravenously in the spinal cat or into the reservoir of blood perfusing the hind leg of a dog, increases the pressor and constrictor action of tyramine and of other amines which act like it, shows that the store which is in or near the vessel wall can be replenished from noradrenaline or adrenaline circulating in the blood. This demonstration appears to indicate that the output of amines from the adrenal medulla may have some importance for maintaining the store in the vessel wall. One of us (Burn, 1932*b*) observed that in the perfused hind leg of the dog sympathetic stimulation became more effective after an infusion of adrenaline, and we have confirmed this observation in the present experiments after an infusion of noradrenaline. We intend to study it further.

From the standpoint of the relation between structure and action the difference in the responses to the two isomers of phenylethanolamine is of interest. While the vascular responses to (+)-phenylethanolamine were similar to those of phenylethylamine, those to (−)-phenylethanolamine were not. In the reserpine-treated animal the latter compound retained a good deal of its pressor action and of its action on the nictitating membrane; moreover, these actions were neither greatly augmented nor greatly depressed by an infusion of noradrenaline. We came to the conclusion that this compound stood midway between 'adrenaline-releasers' like tyramine on the one hand, and adrenaline-like substances on the other. The (+)-isomer, however, was only an 'adrenaline-releaser'. In 1950 Blaschko pointed out the similarity in the potency of (+)-adrenaline and of epinine; he suggested that this similarity was explained by the position of the —OH group in (+)-adrenaline, this position being such that it was unable to exert any effect on the combination between (+)-adrenaline and the receptors; (+)-adrenaline therefore behaved like epinine. The same may be true for (+)-phenylethanolamine, which behaves like phenylethylamine. (−)-Phenylethanolamine, however, has a configuration exactly similar to that of (−)-adrenaline, and therefore approximates to it in behaviour. If this explanation has substance we would expect that (−)-ephedrine would also resemble (−)-phenylethanolamine. This, however, was not found to be so, and ephedrine seemed to be in the main an 'adrenaline-releasing' substance like tyramine. Ephedrine, however, has a —CH_3 group on the α-carbon atom, and perhaps the proximity of this group interferes with the action of the —OH group on the β-carbon atom, so that this group cannot combine with the receptors.

In conclusion, it should be emphasized that the conception of tyramine and similar amines as substances which release adrenaline is a hypothesis only, and it must remain so until a direct demonstration is made. In the perfused rabbit ear, the constrictor effect of tyramine and that of ephedrine, like that of adrenaline and noradrenaline, is reversed by adding tolazoline to the perfusing fluid. The constrictor effect of phenylethylamine and that of amphetamine is, however, not reversed in this way. Yet if the constrictor action of all four substances is due to release of adrenaline or noradrenaline, this action should be reversed by tolazoline in all four cases. We should add that we are also concerned to discover where hydroxytryptamine comes in. We have observed that, in the perfused hind leg of a dog with treated reserpine, the constrictor effect of hydroxytryptamine is reduced by the infusion of noradrenaline. Further, it has been found by Balzer & Holtz (1956) that the pressor action of hydroxytryptamine is somewhat reduced by cocaine. While therefore the observations described in this paper shed new light on old problems, they also reveal problems requiring further study.

SUMMARY

1. When an animal is injected with reserpine the store of noradrenaline-like substance which is present in the vessel wall, and the chromaffin cells adjoining the vessel wall, have been shown to disappear.

2. In the reserpine-treated animal tyramine and many other sympathomimetic amines which are not derivatives of catechol, such as phenylethylamine, ephedrine, amphetamine and (+)-phenylethanolamine, lose their pressor action in the spinal cat and no longer cause contraction of the nictitating membrane or of the volume of the spleen. They also lose their constrictor action in the perfused dog's hind leg.

3. In the reserpine-treated animal, noradrenaline, adrenaline, dopamine, epinine and neosynephrine have, on the other hand, a much greater action than in the normal animal.

4. The action of tyramine and substances acting like it can be restored in the reserpine-treated animal by an infusion of noradrenaline into the blood stream. The restoration is observed after the infusion has stopped and after the rise of pressure which it caused has subsided.

5. When an infusion of noradrenaline has restored the action of tyramine, the actions of noradrenaline, adrenaline, dopamine and neosynephrine are once more reduced to their normal size.

6. The observations suggest that tyramine and similar substances normally act by releasing noradrenaline or adrenaline from the store in the artery wall. They also suggest that there is some continuous small discharge from the store, which diminishes the sensitivity of the vessel wall to injected noradrenaline. This discharge probably plays a part in maintaining vascular tone.

7. The amines liberated from the adrenal medulla into the blood presumably help to maintain the store in the vessel wall.

We wish to thank Miss Roneen Hobbs for making the observations on the perfused rabbit ear, Professor P. Pratesi of the University of Pavia for the two isomeric forms of phenylethanolamine and Dr H. J. Bein of the CIBA Laboratories, Basle, for a supply of reserpine. The work was done by one of us (M.J.R.) during the tenure of a Fellowship awarded by the Life Insurance Medical Research Fund of Australia and New Zealand.

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THE SITE OF INCREASED FORMATION OF HISTAMINE IN THE PREGNANT RAT

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During the last third of pregnancy the rat produces large amounts of histamine, as is indicated by its excretion in the urine (Kahlson, Rosengren & Westling, 1958). In undisturbed pregnancy the urinary excretion of histamine increases from about the 15th day onwards. This increase reaches a peak 1–2 days before parturition and falls steeply to the non-pregnant level at about 1 day before parturition. The amount excreted in 24 hr in some instances was of the same magnitude as the calculated total amount of histamine present in the whole rat. None of the tissues examined in pregnant rats were conspicuously richer in histamine than corresponding tissues in non-pregnant rats. The uterus, placentas and foetuses had a rather low histamine content. In the attempt to identify the tissue responsible for the excessive formation of the amine we were guided by the observation that the urinary excretion of histamine was greater the larger the number of young in the litter.

Three types of experiments are included in the present report. First, the formation will be recorded of ^{14}C-histamine from injected ^{14}C-L-histidine in pregnant and non-pregnant rats. Secondly, the effect of removal of the foetuses on the urinary excretion of histamine will be described. Thirdly, a record is presented of the rate of histamine formation *in vitro* in foetuses and new-born young. A brief report of some of the latter two types of experiments has been given to the Physiological Society (Kahlson, Rosengren, Westling & White, 1958).

METHODS

Animals and drugs. The rats were of the same stock as those used in the experiments already reported, in which the care of the animals, the collection of urine samples and the synthetic histamine-free diet is described (Kahlson, Rosengren & Westling, 1958). In female rats fed on this diet almost all the urinary histamine is in the free form. In some experiments aminoguanidine sulphate (Eastman Kodak) in a dose of 20 mg/kg was injected daily under the skin to diminish the destruction of histamine by histaminase. The duration of pregnancy in the stock of rats used was 22–24 days.

Assay of urinary histamine. Urine was collected in 24 hr samples and free histamine estimated on the guinea-pig's gut as already described (Kahlson, Rosengren & Westling, 1958). The histamine values given in the present paper refer to free histamine expressed as micrograms of the base.

Removal of the foetuses was done under ether anaesthesia. A fairly large mid-line incision was made through the skin of the back and then, on both sides, parallel but shorter incisions were made laterally through the muscles. The left ovary and uterine horn were gently withdrawn and examined, and each foetus was removed through a small hole made opposite to the attachment of its placenta. After replacement of the left ovary and uterine horn the same procedure was performed on the right side. Within 1 hr after the closure of the muscle and skin incisions the rats moved around normally. To see whether the procedures employed in removing the foetuses influenced the urinary excretion of histamine, a sham operation was performed in two pregnant rats: this involved ether anaesthesia and all the procedures mentioned except that no incisions were made in the uterine horns and the foetuses were left in place.

Measurement of histamine formation in vitro. This was done mainly by methods developed in Schayer's laboratory (Schayer, Davis & Smiley, 1955; Schayer, 1956). During a visit of Dr Schayer to our laboratory he modified the original method. The standard technique employed in our laboratory is as follows: Tissues minced with scissors and suspended in the same volume (0·5–2·0 ml.) 0·1 M sodium phosphate buffer of pH 7·4 containing 0·2% glucose were incubated with 40 μg ^{14}C-L-histidine monohydrochloride for 3 hr at 37° C under pure nitrogen (to prevent the action of oxidizing enzymes). This histidine labelled in the 2-position in the imidazole ring has an activity of $9{\cdot}8 \times 10^6$ counts/min/mg base, measured at zero thickness by means of a flow counter. (The radioactive histidine used was synthesized by Dr R. W. Schayer). 1 μg of the labelled histidine quantitatively converted to ^{14}C-histidine dipicrate should give 650 counts/min measured at infinite thickness in a flow counter.

At the end of the incubation 66·4 mg histamine dihydrochloride as carrier and 50 mg L-histidine monohydrochloride as a diluent were added. (Non-isotopic L-histidine was added to dilute the ^{14}C-L-histidine to approximately the same specific activity as the carrier histamine; this reduced the hazard of contamination of the carrier by a minute quantity of histidine of extremely high specific activity). The amount of carrier (66·4 mg) is equivalent to 40 mg of base and 206 mg of the dipicrate. In order to ensure complete mixing the incubation mixture was stirred repeatedly for 15 min. Trichloroacetic acid (TCA) was added to give a final concentration of about 5%. After at least 1 hr the mixture was filtered through paper and the precipitate washed with 5% TCA, after which the TCA was extracted from the filtrate with ether.

To extract the histamine the filtrate was diluted with water to 30 ml. and 3 ml. 11 N-NaOH and 7·5 g anhydrous Na_2SO_4 were added; after this extraction with *n*-butanol was carried out three times with 15 ml. each time for 15 min. All extractions were done using a mechanically driven shaker unless otherwise stated. The combined butanol fractions (45 ml.) were extracted twice with 20 ml. portions of an alkaline solution of L-histidine monohydrochloride (1 mg/ml.). This histidine solution (40 ml.) was extracted twice with 20 ml. portions of butanol for 10 min. All butanol fractions were then combined (45 + 40 = 85 ml.) and extracted by hand-shaking for a few minutes with successive samples of 20 ml. 0·5 N-HCl, 15 ml. 0·1 N-HCl and 15 ml. 0·1 N-HCl. The combined HCl extracts were evaporated to dryness.

Purification of the extracted histamine was carried out by dissolving the residue, consisting mainly of histamine dihydrochloride, in 2 ml. water and adding 180 mg picric acid in 2 ml. absolute ethyl alcohol. The crystals of histamine dipicrate were isolated by filtration (glass-sintered Buchner Funnel, 'Pyrex', capacity 2 ml.), rinsed with cold absolute ethyl alcohol and ether and transferred to a counting plate and counted.

For final purification the histamine was converted into pipsyl histamine. To this end the picric acid was removed by passing the dissolved histamine dipricrate through a small anion exchange column (Dowex-1 in the chloride form). The dried eluate (consisting of histamine dihydrochloride) was dissolved in 2–3 ml. water. To this solution were added 280 mg pipsyl chloride (para-iodobenzenesulphonyl chloride), dissolved in 3 ml. dioxane, and 250 mg sodium bicarbonate.

This mixture was left for 30 min in a mechanical vibrator. Water was gradually added until crystallization was complete. The crystals were isolated by filtration and rinsed with cold alcohol. The crystals of pipsyl histamine were dissolved in hot acetone, active carbon added and the carbon then removed by filtration. The purified pipsyl histamine crystallized on adding water gradually. After filtering, the crystals were rinsed with 25% (w/v) cold ethyl alcohol, transferred to a counting plate and counted. The sample was recrystallized (including treatment with active carbon) until it showed constant radioactivity.

Measurement of radioactivity was made at infinite thickness in a flow counter (98·7% helium and 1·3% butane) on crystals of histamine dipicrate and pipsyl histamine respectively. The sample was mounted on a plate of about 2·5 cm diameter. At least 1000 counts were taken for each sample on a background of 21–24 counts/min. The values given for ^{14}C-histamine, in the form of histamine dipicrate, are counts/min above background.

Determination of urinary ^{14}C-histamine. To each 24 hr sample of urine 66·4 mg histamine dihydrochloride (carrier) and 50 mg L-histidine hydrochloride (diluent) were added. After thorough mixing the sample was subjected to the procedures described above as applied to solutions after TCA had been removed.

RESULTS

Urinary excretion of ^{14}C-histamine after injection of ^{14}C-histidine

After injection of ^{14}C-L-histidine under the skin in rats, ^{14}C-histamine appears in the urine (Schayer, Wu & Smiley, 1954). To see whether the high level of urinary histamine during the last third of pregnancy, as noted by bioassay, is paralleled by increased histidine decarboxylase activity, the urinary excretion of ^{14}C-histamine subsequent to a subcutaneous injection of ^{14}C-L-histidine was determined.

The results are given in Table 1. To reduce the destruction of the formed ^{14}C-histamine, the rats were given the histaminase-inhibitor aminoguanidine, 20 mg/kg once daily, during the whole course of the experiment. 2 mg/kg ^{14}C-L-histidine hydrochloride was injected under the skin at about 9 a.m. and urine was collected for the following three 24 hr periods. In Table 1 the figures for ^{14}C-histamine excreted are given as a fraction of the total amount

TABLE 1. Urinary excretion of ^{14}C-histamine after subcutaneous injection of ^{14}C-histidine 2 mg/kg in pregnant and non-pregnant female rats. The figures within parentheses indicate the day of pregnancy

Rat no.	Weight (g)	Sexual state	^{14}C-histamine excretion (molecules/10^6 molecules histidine injected)		
			1st day	2nd day	3rd day
5	200	Non-pregnant	440	100	70
11	155	5 days after parturition	530	140	90
12	200	Pseudopregnant	640	90	—
15	320	Assumed pregnant, no young born	480	100	50
16	275	Non-pregnant	570	100	60
6	255	Pregnant, 9 young	1720 (19)	—	—
11	165	Pregnant, 8 young	1950 (18)	850 (19)	480 (20)
13	315	Pregnant, 9 young	1370 (18)	280 (19)	230 (20)
14	290	Pregnant, 10 young	1380 (16)	450 (17)	260 (18)

theoretically obtainable from the injected ^{14}C-histidine. To avoid decimals this fraction was multiplied by 10^6. The figures can therefore be said to indicate the number of histamine molecules excreted per 10^6 molecules of injected histidine.

From Table 1 it will be seen that the rats which received injections of histidine during the last week of pregnancy excreted more ^{14}C-histamine than the non-pregnant or pseudopregnant rats. Special mention should be made of rats nos. 14, 15 and 16 which were studied simultaneously. Nos. 14 and 15 had been mated at about the same time and were assumed to be pregnant on the evidence of vaginal smears. In rat no. 14 the urinary histamine, as determined on the guinea-pig's gut, was high (370–670 μg/24 hr) as was the excretion of ^{14}C-histamine. This rat subsequently bore ten young. Rat no. 15, however, behaved differently. The urinary histamine from the 15th day onwards was not increased and the excretion of ^{14}C-histamine was not elevated as compared with the non-pregnant rat no. 16. Eventually rat no. 15 bore no young, indicating that this rat had been either pseudopregnant or that the foetuses had been absorbed in the uterus.

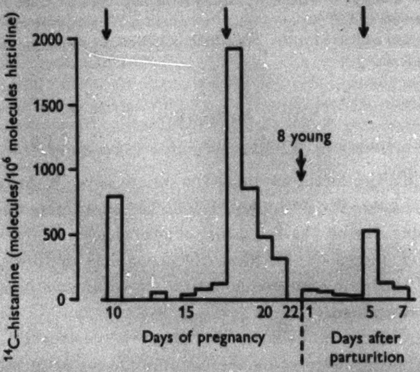

Fig. 1. Urinary excretion of ^{14}C-histamine in rat no. 11, wt. about 160 g. The ordinate represents ^{14}C-histamine expressed as number of histamine molecules excreted per 10^6 molecules of injected histidine. Each arrow at the top of the diagram indicates subcutaneous injection of 330 μg ^{14}C-histidine.

Fig. 1 shows the excretion of ^{14}C-histamine in a female rat (no. 11). Three subsequent injections of the same amount of ^{14}C-histidine were given, the first at the 10th day of pregnancy, the second at the 18th day and the third at the 5th day after parturition (8 young). The second injection of ^{14}C-histidine evoked the largest excretion of ^{14}C-histamine.

The experiments recorded here confirm, by a more direct method, our previous observations of an increased production of histamine during the last week of pregnancy (Kahlson, Rosengren & Westling, 1958).

Urinary histamine after removal of the foetuses

It is known from various reports that if the foetuses are removed deliberately without dislodging the placentas, the rat remains physiologically pregnant by a series of criteria easily recognizable at autopsy (for literature see Newton, 1949). This technique of foetal removal with placental retention appeared useful for the investigation of the part played by the foetuses and the other products of conception in the increased formation of histamine.

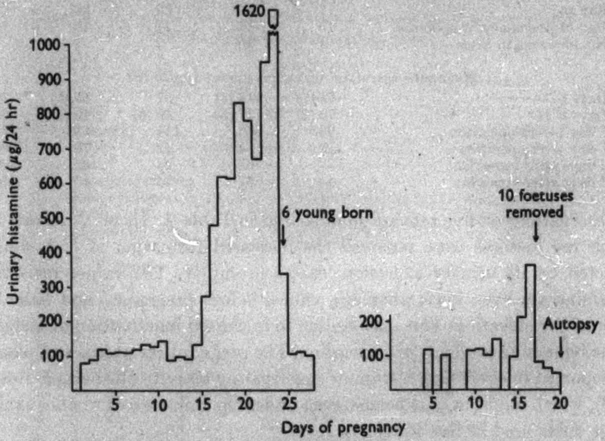

Fig. 2. Urinary excretion of histamine in undisturbed pregnancy (left side of the figure) and in a rat where the foetuses were removed at the 17th day of pregnancy (right side). Throughout the whole course of the observations the rats were under the influence of the histaminase-inhibitor aminoguanidine.

In three rats the foetuses were removed at about the 17th–19th day of pregnancy when increased formation of histamine was indicated by high values for urinary histamine. At autopsy 3 days after removal of the foetuses it was noted that the attributes of pregnancy persisted except for the absence of foetuses: the foetal and maternal placentas were retained, the endometrium was proliferated and the ovaries contained large corpora lutea. In two other rats a sham operation was performed. A typical experiment is shown in Fig. 2. Both rats were given aminoguanidine to reduce the rate of deamination of formed histamine. It will be seen that in undisturbed pregnancy, ni the rat represented in Fig. 2 (left side), the urinary excretion of histamine increases from the 15th day onwards. This increase reaches a peak the day before parturition. On the day of parturition the urinary histamine falls steeply. In the other rat the foetuses were removed at the 17th day of pregnancy, that is, on the second day of increased excretion of histamine and before it had reached its peak value. After removal of the foetuses the urinary histamine rapidly reverted to the pre-pregnant level or even lower.

TABLE 2. Urinary histamine in three rats from which the foetuses were removed and in two rats subjected to sham operation. The figures for histamine excretion followed by parentheses are mean values. The number of observations are given in parentheses. Rat no. 12 was under the influence of aminoguanidine

	Removal of foetuses			Sham operation	
Rat no. ...	11	12	20	25	26
Day of pregnancy at operation	19	17	17	19	18
No. of young in litter	4	10	7	7	7
Histamine excretion during pregnancy (μg/24 hr)					
Days 1–7	68 (2)	113 (2)	57	45 (5)	78 (6)
Days 8–14	70 (2)	117 (5)	49 (6)	40 (6)	60 (7)
1 day before operation	96	365	114	52	177
1 day after operation	22	84	42	78	186
2 days after operation	—	65	24	82	302
3 days after operation	—	51	24	68	216

Observations on five rats are summarized in Table 2. In all three rats from which the foetuses were removed the increased formation of histamine, as reflected by its urinary excretion, ceases promptly. The values for urinary histamine are even lower than the values before pregnancy and before the onset of the elevation. This may be due to increased inactivation of histamine in the remaining products of conception. The pregnant rat uterus and placenta are reported to exert high histamine-inactivating activity (Roberts & Robson, 1953), which might in part persist even under the influence of aminoguanidine in the doses used in this study.

After the sham operation performed in the rats of Table 2 the onset and pattern of increased formation of histamine, as inferred from the urinary content, is essentially the same as in undisturbed pregnancy.

A fourth pregnant rat was operated on at the 17th day of pregnancy with the purpose of removing all the foetuses. The subsequent fall in urinary histamine to the low expected level did not, however, occur. At a second operation 3 days later is was found that out of a total of seven foetuses one had been left in place. After removal of the remaining foetus the urinary histamine rapidly fell below the pre-pregnant level.

From these experiments it appears that the increased formation of histamine during the last third of pregnancy depends on the presence of the foetuses and not on other structures characteristic of pregnancy.

Histamine formation in vitro in the foetus and in various tissues from pregnant and non-pregnant rats

In this part of the study the site of histamine formation was investigated by a more direct approach. Various tissues of the pregnant mother were examined at times when the urinary excretion of histamine was high. Foetuses were removed and examined during the period of high histamine excretion and during the period of decreasing excretion. The histamine formation was also estimated in new-born young. In addition, determinations were made on three non-pregnant female rats. The results are summarized in Table 3 from which it will be seen that in pregnant as well as in non-pregnant rats the stomach is most potent in the formation of histamine, followed next by the lungs, whereas the uterus, the maternal and foetal placentas and the other tissues examined show low activities. In the tissues investigated there was no conspicuous difference between pregnant and non-pregnant female rats as regards histamine formation. The observations on stomach and lung are in agreement with those of Schayer (1956) who found that in the rat the stomach is the most active in decarboxylating ^{14}C-histidine, followed by the lung.

Table 3. Histamine formation in rat tissues, foetuses and new-born young. The figures are counts/min/g tissue

	Non-pregnant females			Mothers	
Lung	89	—*	—	90	150
Stomach†	—	690	450	980	370
Kidney	16	3	—	<4	3
Uterus	<44	5	—	<7	6
Maternal placenta	—	—	—	<10	—
Foetal placenta	—	—	—	<5	9
Brain, total	26	20	35	—	—
Abdominal skin	—	—	—	—	9

Foetuses	Day of pregnancy			New-born	
1.	17	1110	1.	<3 hr old	76
2.	17	2470	2.	<1 hr old	76
3.	17	2360	3.	Collected from the vagina during parturition	210‡ 400
4.	19	1510			
5.	20	2480			
6.	21	290‡ 380	4.		460‡ 970
7.	22	410§			
8.	23	520			
9.	23	1080‖ 1090			

* Not determined. † Total stomach wall. ‡ Different foetuses. § Two foetuses pooled. ‖ Duplicates.

The foetus, during the period of increased urinary excretion of histamine (nos. 1–5 in Table 3), is very potent in producing histamine. The new-born rat, on the day after birth, produces histamine at a low rate. A few observations were made during the time between the expected peak values of histamine excretion and low values at term. The foetuses were removed 1 and 2 days respectively before the expected term. The advent of parturition was recognized by a fall in histamine excretion. The results are summarized in Table 3 (nos. 6–9), from which it will be seen that in these rats the rate of histamine formation in the foetuses appears somewhere intermediate between the peak figures and the low values at term.

Effect of inhibitors of histidine decarboxylase. It appears likely that the formation of histamine by the foetus under the present conditions results from the action of histidine decarboxylase. Accordingly, the effects of two inhibitors of this enzyme, semicarbazide and hydrazine, were studied in a few experiments. Three foetuses were minced in one pool and divided into six portions, two serving as controls. The samples were incubated with ^{14}C-histidine as described above and the formed ^{14}C-histamine was measured. The control samples yielded approximately 1600 counts/min/g. With hydrazine sulphate in the concentration 10^{-4}M in the incubation mixture the corresponding counts in duplicate determinations were approximately 280, and with semicarbazide hydrochloride $0{\cdot}6 \times 10^{-3}$M the counts were approximately 90.

DISCUSSION

It was shown recently (Kahlson, Rosengren & Westling, 1958) that in the rat, at about the 15th day of pregnancy, a distinct and steep rise occurred in the urinary excretion of histamine with peak values at 1–2 days before the birth of the young. On the day before parturition the histamine excretion fell steeply towards the pre-pregnant level. Yet the content of histamine in the mother's body did not change appreciably during pregnancy. It was noted further that the larger the number of young in the litter the greater the increase in histamine excretion, and this suggested that an excessive formation of histamine during the last third of pregnancy takes place in the uterus and its contents.

The present experiments show more directly that the excessive formation of histamine during the last third of pregnancy is due to an increase in the rate of histidine decarboxylase activity. During the last third of pregnancy injected ^{14}C-histidine is decarboxylated at a much higher rate than in non-pregnant females as indicated by the urinary excretion of ^{14}C-histamine.

Our experiments further demonstrate that the foetus is the site of the increased histamine formation. Removal of the foetuses with the least possible interference with the subsequent course of pregnancy regularly and rapidly abolished the excessive excretion of histamine. Since in the rat removal of the foetuses does not interrupt the further course of 'pregnancy' it appears that among the uterus and its contents the foetus is the sole structure responsible for the excessive histamine formation.

The experiments on histamine production *in vitro* give information on three points. First, they show in a most direct way that foetal tissue decarboxylates histidine at a very high rate. The rate of histidine decarboxylase activity of the foetus even exceeds that of the stomach which is richer in histidine decarboxylase than any other tissue investigated in the rat. Secondly, the *in vitro* experiments reveal that during the last 1–2 days of gestation the rate of histamine formation in the foetus regresses towards a level which, in the newborn, is low as compared with the peak level. Lastly, the experiments show a relationship between foetal histamine formation and urinary histamine excretion. This finding supports the claim that in the female rat raised on a synthetic histamine-free diet the urinary excretion of free histamine faithfully reflects the rate of endogenous histamine production (Gustafsson, Kahlson & Rosengren, 1957).

The rat foetus does not contain much histamine. At the 19th-20th day of gestation, when the histidine decarboxylase activity and the urinary histamine excretion are at peak levels, the foetus contains about 3–5 μg/g tissue. At this stage the excess histamine produced by a set of 6–10 foetuses in 24 hr may even equal the total amount of histamine present in the whole body of the mother rat (Kahlson, Rosengren & Westling, 1958). A foetus weighing 2·5 g and containing about 10 μg histamine may produce 100 μg of the amine in 24 hr. In the relationship between rate of secretion and actual content of the elaborated product the foetus resembles certain endocrine organs, e.g. the adrenal cortex.

No attempt was made in the present study to examine whether and at what rate the embryo produces histamine during the 2 first weeks of gestation. By the methods used the excess production is manifest from about the 15th day onwards. At this day the single foetus has an average weight of 0·35 g, increasing within the short period of a week to about 5 g at term. At the 9th day of gestation the embryo weighs 0·006 g, at the 11th day 0·02 g and at the 14th day 0·27 g (Misrahy, 1946). It appears that from the 15th day onwards until about the 20th day the formation of histamine, as reflected by its urinary excretion, very roughly corresponds to the increase in weight of the foetuses during this period. It is noteworthy that in no instance was increased excretion observed before the 15th day, not even in animals given a histaminase inhibitor. This is suggestive of a low rate of histamine formation until the 15th day of pregnancy. This day marks the beginning of a large daily gain in weight of the foetuses which, from the 15th to the 16th day, increase in weight by about as much as during the previous 2 weeks. In this connexion it is of interest to note that in the developing egg of the hen, where the duration of embryonic development is about the same as in the rat, the histamine concentration of the embryo increases tenfold between the 13th and the 19th day (Misrahy, 1946).

Whereas the time of the first appearance of high histidine decarboxylase activity in the embryo is uncertain, the time of its disappearance in the foetus is rather well defined. About 1 day before term the high enzyme activity subsides. The factors responsible for the timing of this event and for the loss of enzyme activity are unknown. The time clock controlling the rate of histamine formation might be linked up with the machinery which controls the duration of gestation.

There is evidence of a regulatory mechanism controlling the concentration of histamine in the gastro-intestinal tract where the histamine content is not correlated to the number of mast cells. Haeger, Kahlson & Westling (1953) found that in cats the concentration of histamine is uniform in the stomach wall of the mother and her foetuses and also in the intestinal wall among foetuses of the same mother, whereas after birth the young go their own way and establish individual levels of histamine scattered within the wide range of variation characteristic of the feline species. This was taken to indicate the existence of a regulatory mechanism operating via the blood stream. Little is known about this, except that removal of the pituitary gland or the adrenals does not influence the concentration of histamine in the gastro-intestinal tract (Haeger, Jacobsohn & Kahlson, 1952).

Within the family of enzymes involved in histamine metabolism it has been shown that the activity of histaminase and histidine decarboxylase is influenced by adrenocortical hormones. In cats, even 6–7 hr after adrenalectomy, at least 75 % of the histaminase activity of the whole body disappears (Haeger & Kahlson, 1952). As to histidine decarboxylase, pre-treatment of rats for 3 days with cortisone or its analogues causes a reduction to less than half of the histidine decarboxylase activity of the lung (Schayer, 1956). The actual level of activity of these enzymes seems to depend on factors which operate within the normal physiological range. The analogy to the sudden loss of enzyme activity in the foetus before term is merely on principle. It should be recalled that the fall in the foetus is on a much larger scale. Even this scale holds under the unlikely proposition that the enzyme is uniformly produced by all tissues of the foetus. It appears more likely that the increased histamine formation takes place in certain tissues yet to be identified. In this case the loss of activity before term would appear on a still more remarkable scale. Only further studies can show whether the striking change in histamine formation is due to diminished rate of synthesis of histidine decarboxylase, the appearance of an inhibitory factor, the disappearance of an activator or other circumstances.

Another relationship between histamine and pregnancy is indicated by the appearance of a high histaminase activity in the placenta. This occurs in several species but seems to be most pronounced in man and the rat. The histaminase resides mainly in the maternal parts of the placenta (Swanberg, 1950). In the rat even the uterine muscle is rich in histaminase (Roberts & Robson, 1953). As to the function of placental and uterine histaminase it is believed that it protects the foetus and the uterus against deleterious actions of histamine. Since, at least in the rat, the foetus produces much histamine, it appears rather that the mother requires protection against histamine excreted by the foetus: the placental histaminase may provide a barrier between the foetus and the circulation of the mother. The functional contact between histamine leaving the foetus and placental histaminase is, however, not efficient enough to prevent considerable amounts of histamine from entering the mother's circulation, whence it is excreted in the urine. Even without aminoguanidine large amounts of histamine formed in the foetus appear in the urine. As to the function of histamine produced by the foetus, it would seem on *a priori* grounds to lie within the foetus and/or the placenta. Further information, in particular from other animal species, is required on this point.

SUMMARY

1. The excess formation of histamine in pregnant rats was investigated by three different criteria: (*a*) the urinary excretion of ^{14}C-histamine after subcutaneous injection of ^{14}C-L-histidine; (*b*) the effect of removal of the foetuses on the urinary excretion of histamine; and (*c*) histamine formation from ^{14}C-L-histidine *in vitro*.

2. During the last third of pregnancy the rate of formation of ^{14}C-histamine from injected ^{14}C-histidine is greatly increased as indicated by the urinary excretion of ^{14}C-histamine.

3. Removal of the foetuses without otherwise interfering with the course of pregnancy abolishes the increased urinary excretion of histamine.

4. The rate of histamine formation *in vitro* in foetuses removed at the 17th–20th day of gestation exceeds that in any other tissue examined. At about one day before term a fall occurs in the rate of histamine formation in the foetus. The formation of histamine by the foetus *in vitro* is inhibited by hydrazine sulphate and semicarbazide hydrochloride, inhibitors of histidine decarboxylase.

5. It is concluded that in rat pregnancy the foetuses produce large amounts of histamine which escapes into the mother's circulation and raises her urinary excretion of histamine.

We are grateful to Dr R. W. Schayer who during a long stay at our laboratory most generously instructed us in his methods. A. B. Ferrosan, Malmö, Sweden, kindly supplied vitamins for the synthetic diet. The present investigation was supported by a grant from the Rockefeller Foundation.

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ANTAGONISM BY γ-AMINOBUTYRIC ACID TO THE ACTIONS OF 5-HYDROXYTRYPTAMINE AND NICOTINE ON ISOLATED ORGANS

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γ-Aminobutyric acid (GABA) is a normal constituent of mammalian brain which on topical application reverses surface-negative post-synaptic potentials of the apical dendrites of the cerebral cortex (Purpura, Girado & Grundfest, 1957*a*, *b*; Iwama & Jasper, 1957) and blocks transcallosal conduction when injected into the internal carotid artery (Marrazzi, Hart & Rodriguez, 1958). The mechanism by which these effects are produced is unknown. Thus studies of the actions of GABA on comparatively simpler neuronal structures provide an alternative approach by which useful information might be gained.

For this reason the effects of GABA on the isolated ileum of the guinea-pig, rabbit and rat were investigated (Hobbiger, 1958). In these experiments it was found that GABA produced a variety of effects which were more pronounced and more consistently reproducible on the guinea-pig ileum than on the rabbit or rat ileum. On the guinea-pig ileum GABA to a limited extent antagonized nicotine, and under certain experimental conditions acetylcholine and histamine when the stimulant drugs were used in concentrations which produced a submaximal shortening of the longitudinal muscle. The antinicotine effect of GABA appeared to be of greater importance than its antiacetylcholine or antihistamine effect, and for this reason the former was selected for further investigation.

This paper describes the antagonism of GABA to 5-hydroxytryptamine (5-HT) on the guinea-pig ileum. The experimental evidence presented indicates that GABA antagonizes nicotine and 5-HT by a mechanism which operates outside the 'receptor' sites. The antagonism of GABA to nicotine and 5-HT has many features in common with the antagonism of morphine to the same substances. GABA and morphine are most effective on the guinea-pig ileum and the antagonistic action of GABA is considerably reduced by morphine. In preparations in which morphine has no anti-5-HT effect only very little or no anti-5-HT effect is obtained with GABA.

METHODS

Isolated organs. These preparations were obtained from female guinea-pigs weighing 150–250 g, male and female rabbits weighing 1·5–3 kg and male and female Wistar rats weighing 150–200 g. A modified Tyrode solution was used which contained (%, w/v): NaCl 0·9, KCl 0·02, $CaCl_2$ 0·02, $MgCl_2$ 0·01, $NaHCO_3$ 0·05 and glucose 0·1, and was aerated with 95% O_2 and 5% CO_2. The temperature at which the experiments were carried out was 37° C in the case of the isolated ileum of the guinea-pig or rabbit and the isolated duodenum of the rat and 27–31° C in the case of the isolated uterus of the guinea-pig or rat. Changes in tone exerted in the longitudinal direction were recorded on a smoked drum by means of an isotonic writing lever (amplification 8- to 12-fold), the volume of the bath in which the isolated organs were suspended was kept constant at 8 ml. and stimulant drugs were used at 4 min intervals. Unless otherwise stated the stimulant drugs were allowed to act for 30 sec, by which time a maximum effect had developed. The organ bath was then drained and refilled with fresh, pre-warmed and oxygenated Tyrode solution; the change of Tyrode solution was repeated 45 sec later. Unless otherwise stated, only the tone of the isolated organ preparation over a period of 1 min preceding the addition of a stimulant drug to the organ bath and a period of 30 sec following the latter was recorded.

Antagonistic effects were evaluated either as percentage reduction in sensitivity (Hobbiger, 1958, and also text of Fig. 1 and Table 1) or as differences between responses obtained by a given concentration of the stimulant drug in the presence and absence of the antagonist.

Isolated rat phrenic nerve-diaphragm preparation. This preparation was set up in the conventional manner (Burgen & Hobbiger, 1951) using as bath fluid the Tyrode solution described above.

Throughout the text final concentrations in the organ bath are given in preference to quantities added. Acetylcholine was used as chloride, nicotine as hydrogen tartrate and 5-hydroxytryptamine as creatinine sulphate. For simplicity these substances are referred to by the name of the base only but all concentrations given refer to the salts. For 5-hydroxytryptamine and γ-aminobutyric acid (Merck or Roche Products Ltd.) the abbreviations 5-HT and GABA respectively are used.

RESULTS

Guinea-pig ileum

GABA in concentrations of 10–100 μg/ml. reduces the sensitivity of the ileum to nicotine by 35–75% when added to the organ bath 20 sec before a dose of nicotine, which in the absence of GABA causes a 5–15% shortening of the longitudinal muscle (Hobbiger, 1958). It has now been shown that under comparable conditions GABA was more effective as an antagonist of 5-HT than of nicotine if the reduction in sensitivity of the ileum to the stimulant drug is used as the measure of the antagonistic action of GABA (Table 1).

Although the degree of 5-HT antagonism obtained with a given concentration of GABA varied in different experiments, in any individual experiment graded degrees of antagonism were obtained over a 100-1000-fold range of GABA concentrations (Fig. 1). In some experiments a maximum effect was reached with 10 μg/ml. GABA but in others 100 μg/ml. GABA gave a slightly greater effect than 10 μg/ml. GABA. The latter result occurred most frequently with those preparations in which 10 μg/ml. GABA gave the least effect.

With all concentrations of GABA a maximum effect was reached quickly, and lengthening the interval between the administration of GABA and 5-HT

Table 1. The antagonism between GABA and 5-HT on guinea-pig ileum

GABA (μg/ml.)	5-HT antagonism of GABA expressed as % reduction in sensitivity
0·01	0
0·1	10–50
1	50–87·5
10	75–95

GABA was added to the organ bath 20 sec before 5-HT. The figures given cover the entire range of individual results obtained with fresh segments of ileum taken from twenty-six guinea-pigs. % reduction in sensitivity = (100 − [100/x]) %, where x is the ratio of 5-HT concentrations causing equal contractions in the presence and absence of GABA; x is identical with the dose ratio of Gaddum & Picarelli (1957). With values of 2, 4, 8 and 16 for x, the % reductions in sensitivity are 50, 75, 87·5 and 93·75 respectively.

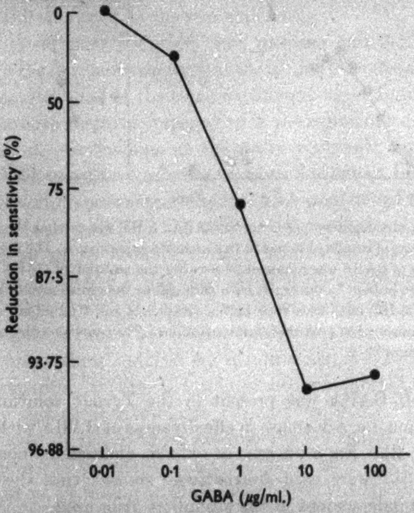

Fig. 1. GABA–5-HT antagonism observed in a typical experiment on the guinea-pig ileum. GABA was added to the organ bath 20 sec before 5-HT. The antagonistic effect of GABA is plotted on an arbitrary scale as a % reduction in sensitivity of the ileum to 5-HT. % reduction in sensitivity = (100 − [100/x]) %, where x is the ratio of 5-HT concentrations causing equal contractions in the presence and absence of GABA. For further explanation see Table 1.

from 20 sec to 2 min resulted in either identical or very similar degrees of antagonism. After removal of GABA from the organ bath recovery of the pre-GABA sensitivity of the ileum to 5-HT was always very quick and identical degrees of antagonism were obtained if a given concentration of GABA was used at 12–16 min intervals. A transient increase in sensitivity of the ileum to 5-HT was often seen after GABA had been used in concentrations above 1 μg/ml. No increase in the effectiveness of GABA resulted from prolonged storage of the ileum at room temperature or 4° C before use, a procedure which markedly enhances the antiacetylcholine and antihistamine effect of GABA (Hobbiger, 1958).

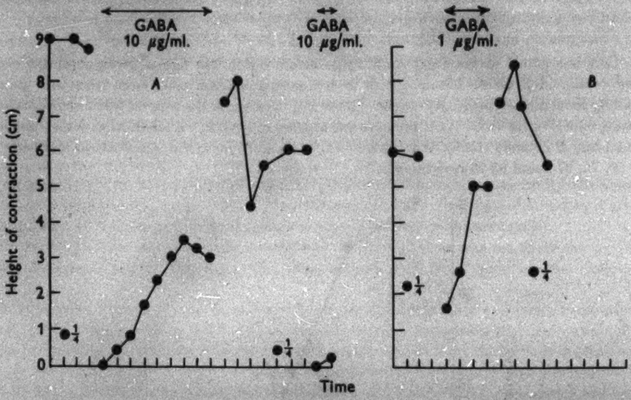

Fig. 2. Adaptation of the guinea-pig ileum to GABA. 5-HT was used as the stimulant drug and *A* and *B* are plots of results obtained in two separate experiments. The first response to 5-HT in the presence of GABA was obtained 20 sec after the addition of GABA to the organ bath; GABA was then present in the organ bath throughout the entire period indicated. The concentrations of 5-HT used were 0·01 μg/ml. (Expt. *A*) and 0·02 μg/ml. (Expt. *B*); ● ¼ are responses obtained with ¼ of these concentrations. The marks on the time scale represent intervals of 4 min.

Adaptation. If GABA was present in the Tyrode solution for 5 min or longer, adaptation, i.e. a decrease in effectiveness of GABA with time, occurred regularly (Fig. 2). Experiments in which equivalent concentrations of nicotine and 5-HT were used alternatively showed that the time course of adaptation was independent of the stimulant drug used.

Qualitatively these findings resemble closely those obtained in studies of the GABA–nicotine antagonism (Hobbiger, 1958). Thus further investigations were undertaken to find out whether a single mechanism could account for the antagonism of GABA to nicotine and 5-HT.

Studies of the mechanism responsible for the GABA–nicotine and GABA–5-HT antagonism. It is now well established that on the guinea-pig ileum morphine reduces the effectiveness of stimulation of nervous structures but has no effect when substances are used which trigger a contraction by interacting with the smooth muscle fibres themselves (Schaumann, 1957; Paton, 1957; Kosterlitz, Robinson & Taylor, 1957). To locate the site of action of 5-HT under the experimental conditions, morphine sulphate, in a final concentration of 0·0025–0·1 μg/ml., was added to the organ bath 20 sec before nicotine and 5-HT respectively. The latter were used in concentrations which gave equal effects in the absence of morphine. Altogether eight experiments were carried out and the antagonistic action of morphine was assessed by comparing the heights of contractions obtained with the same concentration of the stimulant drug in the absence and presence of morphine. In five of these experiments a given concentration of morphine antagonized nicotine and 5-HT to the same extent and in three experiments morphine was slightly more effective against nicotine than against 5-HT.

On the other hand, pentamethonium bromide, in a concentration of 100 μg/ml., markedly antagonized the effect of nicotine but was devoid of such an action if 5-HT was used as the stimulant drug.

These findings support the view that nicotine and 5-HT interact with different sites of the nervous structures of the ileum. The interaction leads to the discharge of acetylcholine at the same cholinergic nerve terminals. If GABA interferes either with impulse propagation in the region of a common pathway or with release of acetylcholine at the nerve terminals, equal responses to nicotine and 5-HT should be affected to a similar extent by GABA. To test this hypothesis various concentrations of GABA were added to the organ bath 20 sec before nicotine or 5-HT. The concentrations of nicotine and 5-HT used were matched so as to produce similar heights of contractions, and the antagonistic effect of GABA was then assessed by comparing the heights of contractions obtained with the same concentration of the stimulant drug in the presence and absence of GABA. A given concentration of GABA antagonized contractions induced by nicotine and 5-HT to a very similar extent in ten experiments (Fig. 3), but in six experiments GABA was definitely more effective against contractions induced by 5-HT. The greatest discrepancy observed in any of these experiments is illustrated in Fig. 4.

To investigate the possibility that morphine and GABA produced their antagonistic effects by acting at the same receptor site the following approach was used. The GABA antagonism was assessed using first normal Tyrode solution and then a Tyrode solution containing 0·02 μg/ml. morphine sulphate. The concentration of morphine chosen reduced the sensitivity of the ileum to nicotine and 5-HT by the same amount as 10–100 μg/ml. GABA or more. As can be seen from Fig. 5, which is a record of a typical experiment, morphine

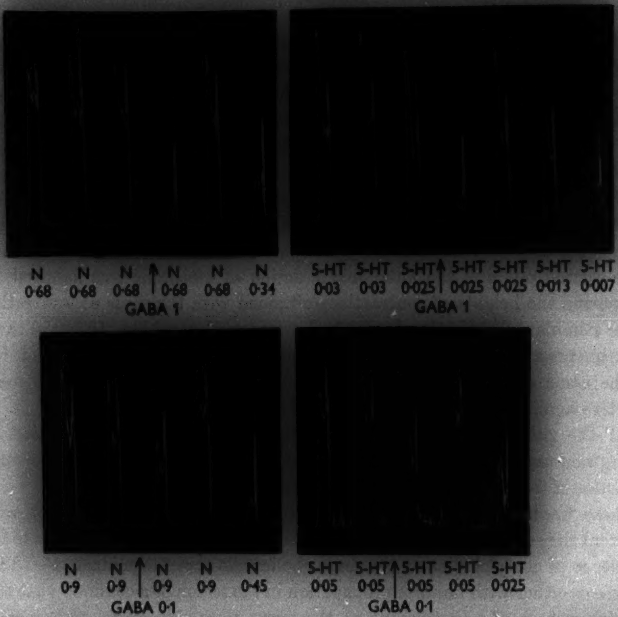

Fig. 3. Comparison of the GABA–nicotine and GABA–5-HT antagonism on the guinea-pig ileum. GABA was added to the organ bath 20 sec before nicotine (N) and 5-HT respectively; figures represent concentrations in μg/ml. In both experiments the discontinuity of the record represents a period of not more than 12 min during which a suitable concentration of 5-HT was established.

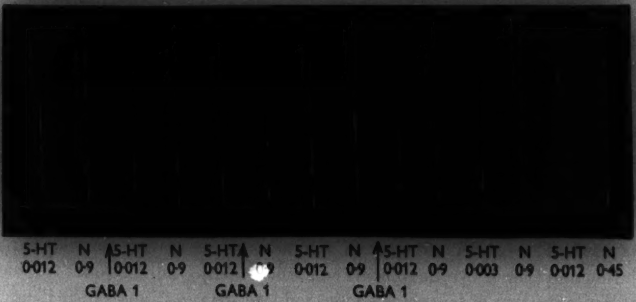

Fig. 4. Comparison of the GABA–nicotine and GABA–5-HT antagonism on the guinea-pig ileum. N indicates nicotine and figures represent concentrations in μg/ml. GABA was added to the organ bath 20 sec before the stimulant drug.

considerably reduced the effectiveness of GABA. If, on the other hand, the morphine antagonism was assessed using first normal Tyrode solution and then a Tyrode solution containing 10 μg/ml. GABA the reduction in the effectiveness of morphine under the latter conditions was always far less marked than the reduction in effectiveness of GABA achieved with morphine.

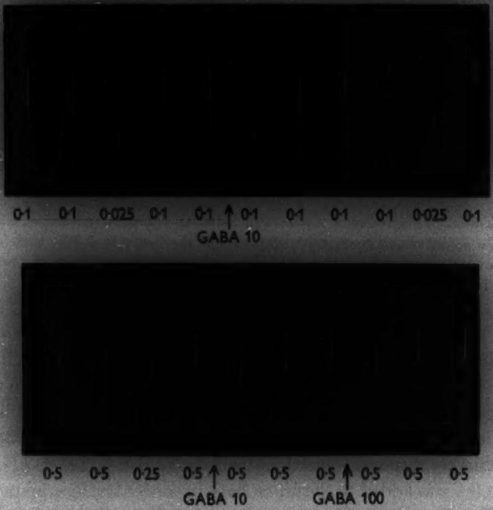

Fig. 5. Effect of morphine on the GABA-5-HT antagonism on the guinea-pig ileum. The upper record shows the effect of GABA on the ileum while normal Tyrode solution was used. The experiment was then continued using a Tyrode solution containing 0·02 μg/ml. morphine sulphate (lower record). GABA was added to the organ bath 20 sec before the stimulant drug; figures represent concentrations in μg/ml. The same results were obtained when nicotine was used as the stimulant drug.

Rabbit ileum

A GABA–5-HT antagonism was seen in each of three experiments in which an isolated rabbit ileum was used. However, the effect of GABA on the rabbit ileum was small compared with its effect on the guinea-pig ileum. The antagonism produced by GABA corresponded to a reduction in sensitivity by not more than 50% when 10 or 100 μg/ml. GABA was added to the organ bath 20 sec before 5-HT (Fig. 6). A similar species dependence of effectiveness could be demonstrated for morphine, which in concentrations of 0·02–0·2 μg/ml. (as sulphate) had no effect on the sensitivity of the rabbit ileum to 5-HT. The latter finding is in agreement with the observations of Medaković (1958).

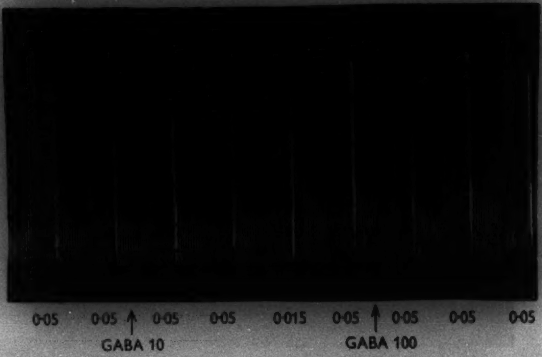

Fig. 6. GABA–5-HT antagonism on the rabbit ileum; figures represent concentrations in μg/ml. The record shows consecutive responses of the ileum to 5-HT, each of which was recorded over a period of 1 min, together with the base line preceding it, also recorded over a period of 1 min. GABA was added to the organ bath 20 sec before 5-HT.

Rat duodenum

In previous studies (Hobbiger, 1958) no measurable GABA–nicotine antagonism was observed when the isolated rat ileum was used. In the current series of experiments the duodenum was used in preference to the ileum, since the former is not only extremely sensitive to 5-HT (Erspamer, 1954), but also gave reproducible responses over long periods.

When GABA was added to the organ bath in concentrations up to 100 μg/ml. before 5-HT no GABA–5-HT antagonism was observed in eight experiments (Fig. 7). When, on the other hand, GABA in concentrations of 1–100 μg/ml. was added to the organ bath after 5-HT a transient relaxation of the 5-HT-induced spasm was seen regularly (Fig. 7). This transient relaxation, however, was not the result of a selective GABA–5-HT antagonism, since identical degrees of relaxation were also observed when acetylcholine was used instead of 5-HT.

As in the case of the rabbit ileum, morphine sulphate in concentrations of 0·02–0·2 μg/ml. had no effect on the sensitivity of the rat duodenum to 5-HT.

Rat uterus and guinea-pig uterus

To see if any GABA–5-HT antagonism could be demonstrated on organs in which the stimulant effect of 5-HT was also entirely the result of an interaction of 5-HT with smooth muscle fibres only (Gaddum & Hameed, 1954), the effect of GABA on the 5-HT-induced spasm of the uterus was investigated.

Rats and guinea-pigs received on 3 consecutive days a single daily intramuscular injection of 5 μg/100 g stilboestrol in arachis oil. Such pre-treatment was given because the sensitivity of the uterus to 5-HT is highest during oestrus (Gaddum & Hameed, 1954; Erspamer, 1954).

In twelve experiments in which GABA in concentrations up to 100 μg/ml. was added to the organ bath 20 sec before 5-HT, only a very small degree of GABA–5-HT antagonism, corresponding to a reduction in sensitivity by less than 25 %, was seen occasionally.

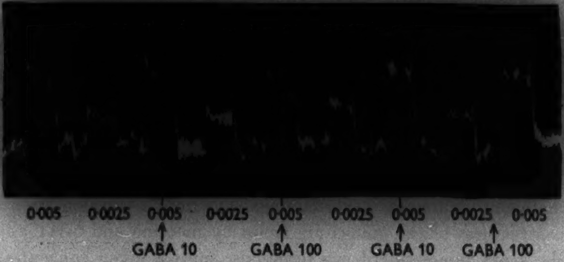

Fig. 7. GABA–5-HT antagonism on the rat duodenum. GABA was added to the organ bath either 30 sec after 5-HT (first three applications of GABA) or 20 sec before 5-HT (last application of GABA). The record shows consecutive responses of the duodenum to 5-HT, each of which was recorded over a period of 1 min, together with the base line preceding it, also recorded over a period of 1 min; all figures represent concentrations in μg/ml.

Local anaesthetic action of GABA

The GABA–5-HT and GABA–nicotine antagonism on the guinea-pig ileum could be explained if GABA had local anaesthetic properties. That this is not the case is shown by the following experiments.

GABA was injected intradermally in a volume of 0·1 ml. into the skin of the human forearm and the sensitivity of the injected area to a pin-prick which was painful for normal skin was investigated. Two subjects were used and in both solutions containing 10 or 100 mg/ml. GABA occasionally dulled perception of pain only slightly more than control injections of saline. In agreement with this is the finding that 1–2 drops of a solution containing 10 mg/ml. GABA when instilled into the conjunctival sac of a rabbit's eye had no local anaesthetic action.

GABA in concentrations up to 100 μg/ml. also had no effect on the response of the *isolated rat diaphragm* to indirect stimulation by supra- and submaximal electrical stimuli applied to the phrenic nerve.

DISCUSSION

The experiments described in this paper show that GABA is only a very feeble antagonist of 5-HT on the rabbit ileum, rat duodenum, rat uterus and guinea-pig uterus. The stimulant effect of 5-HT on these organs is mainly if not entirely the result of an action of 5-HT on smooth muscle fibres and morphine does not interfere with it.

The effect of GABA on the guinea-pig ileum is an exception, for the antagonism obtained with GABA on this preparation is considerably greater. A 5- to 20-fold increase in the concentration of 5-HT is required to compensate for the effect of 10–100 μg/ml. GABA, whereas under the same conditions only a twofold or even smaller increase in the concentration of 5-HT is needed in the other preparations. 5-HT causes a shortening of the longitudinal muscle of the guinea-pig ileum as a result of its interaction with nervous structures; on the same preparation nicotine also acts on nervous structures but at a different site (Robertson, 1953; Roche e Silva, Valle & Picarelli, 1953; Gaddum & Hameed, 1954; Gaddum & Picarelli, 1957).

Schaumann (1957) and Paton (1957) have shown that morphine antagonizes the effect of stimulation of nervous structures by reducing the output of acetylcholine at cholinergic nerve-endings. The impulses which are set up in nervous structures of the guinea-pig ileum by nicotine and 5-HT appear to reach the same cholinergic nerve terminals, since a given concentration of morphine reduces the magnitude of responses by similar extents when nicotine or 5-HT is used in concentrations which are equivalent in the absence of morphine. GABA, like morphine, antagonizes the stimulant action of both nicotine and 5-HT. The antagonism by GABA has many characteristics in common with the antagonism produced by morphine. GABA, like morphine, is devoid of a local anaesthetic action, and the experimental evidence indicates that there is some connexion between the actions of the two drugs. This interpretation is based on the finding that in the presence of morphine, in concentrations which give the same or a greater effect than GABA, the antagonistic action of GABA is considerably reduced. Morphine and GABA, however, do not seem to share a common receptor site, since GABA reduces only slightly the effectiveness of morphine. In some experiments the antagonism to 5-HT is more marked than the antagonism to nicotine, whereas no such tendency is seen when morphine is used. The cause of these discrepancies is obscure at the moment.

One important feature brought out by the experiments described in this paper is the finding that a considerable degree of antagonism is obtained with morphine and GABA only on the guinea-pig ileum, whereas both substances have either no, or only very little, antagonistic action on intestinal preparations of other species or on the uterus of the rat and guinea-pig. Schaumann, Giovannini & Jochum (1952), Paton (1957) and Medaković (1958) demonstrated for morphine and related drugs a close parallelism between *in vivo* analgesic potency and effectiveness in blocking neuronal structures of the guinea-pig ileum. Since GABA does not block cholinergic nerves indiscriminately, as is shown by its failure to affect transmission in an efferent somatic nerve, another property shared with morphine (see also Paton, 1957), the antagonistic effects of GABA on the guinea-pig ileum might perhaps serve as model experiments and help in the interpretation of the effects of GABA on the central nervous system. Such an approach appears to have merits, since some actions of GABA obtained with crayfish preparations, on which most of the work with GABA or GABA-containing Factor I preparations has been carried out, are undoubtedly not applicable to mammalian structures. This is clearly illustrated by the finding that GABA has no effect on mammalian neuromuscular transmission although it inhibits neuromuscular transmission in crustacea (McLennan, 1957).

SUMMARY

1. γ-Aminobutyric acid (GABA) is an antagonist of 5-hydroxytryptamine (5-HT) and of nicotine on the isolated guinea-pig ileum. The antagonism of GABA to nicotine and 5-HT on this preparation appears to be the result of an action of GABA on those neuronal structures which are involved in the transmission of impulses generated by both the stimulant drugs. The antagonism produced by GABA has many characteristics in common with the antagonism produced by morphine. Morphine greatly reduces the effectiveness of GABA, whereas GABA interferes with the action of morphine to a much smaller extent.

2. GABA is either only a feeble antagonist of 5-HT or, like morphine, lacks antagonistic properties towards 5-HT on the rat duodenum, rabbit ileum, rat uterus, guinea-pig uterus and rat phrenic nerve–diaphragm preparation.

3. GABA has no local anaesthetic action.

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INFLUENCE OF THE THYROID ON THE SYNTHESIS OF CHOLESTEROL BY LIVER AND SKIN *IN VITRO*

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Rosenman, Byers & Friedman (1952) have shown that the rate of secretion of cholesterol in the bile of rats is increased by injections of thyroxine and is reduced by removal of the thyroid gland. It has also been shown that the rate of regeneration of the plasma cholesterol in humans is reduced in myxoedema (Kurland & Lucas, 1955; Gould, LeRoy, Okita, Kabara, Keegan & Bergenstal, 1955).

We thought that a study of the synthesis of cholesterol by tissues taken from thyroxine-treated or thyroidectomized animals might give more direct evidence about the influence of the thyroid on cholesterol metabolism. Since acetate is known to provide the carbon atoms of cholesterol, we have used ^{14}C-labelled acetate to measure the rate of synthesis of cholesterol. Acetate is also a precursor of fatty acids. We have therefore measured the rate of incorporation of the labelled acetate into fatty acids, and so have been able to study the influence of the thyroid on two biosynthetic processes which share a common precursor.

Tavormina, Gibbs & Huff (1956) have shown that mevalonic acid (3-hydroxy-3-methyl-pentano-5-lactone) is converted to cholesterol by rat's liver *in vitro*. Since the efficiency of this conversion to cholesterol is much greater than that of acetate, mevalonic acid is generally assumed to be an intermediate in the biosynthetic chain from acetate to cholesterol. We have therefore studied the influence of the thyroid on the synthesis of cholesterol from labelled mevalonic acid, as well as from labelled acetate. In this investigation the synthesis of cholesterol was measured in slices of liver and skin taken from rats, rabbits and guinea-pigs after destruction of the thyroid or injections of thyroxine.

METHODS

The rats were young male albinos; the rabbits were males of the Himalayan breed. A few albino guinea-pigs of both sexes were also used. When killed, the rats weighed about 200 g, the rabbits about 2 kg and the guinea-pigs 600–800 g.

Destruction of the thyroid. The thyroid was destroyed by an intramuscular injection of ^{131}I given as iodide (rats, 1·0 mc; rabbits, 1·5–2·0 mc; guinea-pigs, 1·5 mc). At the time of the injection the rats were aged 2 months, the rabbits 2 months, and the guinea-pigs 1 month. The animals were killed at intervals varying from 2 to 4 months after the injection. At about 6 weeks after the injection, thyroid function was tested by measuring the uptake into the thyroid of a tracer dose of radio-iodide (10–20 μc) given intraperitoneally. The amounts of ^{131}I in the neck and thorax were measured *in vivo* at 24 hr by means of a lead-shielded scintillation counter. When the amount in the neck was less than the amount in the thorax, it was assumed that the thyroid had ceased to function. In normal animals, the neck: thorax ^{131}I ratio at 24 hr was in all cases greater than 7. If the result of the test was doubtful, a second large dose of ^{131}I was given. The pituitaries from four pairs of treated and untreated rats were removed after death, fixed in Helly's fluid, and stained by the method of Pearse (1950). The pituitaries from all four treated rats showed enlargement of the pars anterior, with hyperplasia of the basophil cells and loss of granules from the acidophil cells. The pituitaries of the untreated rats were normal.

Treatment with thyroxine. Sodium-L-thyroxine dissolved in less than 0·5 ml. of dilute Na_2CO_3 was given daily by intramuscular injection for 10 days. Control animals were injected with the same volume of a similar solution of Na_2CO_3 containing no thyroxine. After daily injections of 250 μg of thyroxine for 10 days the rabbits became wasted and excitable, and a few of them died. None of the rats showed any obvious ill effects after injections of 500 μg/day for 10 days.

Incubation medium and radioactive precursors. The slices were incubated in Krebs's phosphate buffer (10 ml./g tissue) at pH 7·4, modified by equalizing the concentrations of sodium and potassium ions. The two labelled precursors were: [1-^{14}C]-sodium acetate (obtained from the Radiochemical Centre, Amersham) and DL-3-hydroxy-3-methyl-[2-^{14}C]-pentano-5-lactone (Cornforth, Cornforth, Popják & Gore, 1958). The ^{14}C-acetate was added as isotonic acetate in sufficient quantity to give approximately 40 μmoles/g tissue. The specific activity of the acetate when added to the flasks was about 0·1 μc/μmole. Enough radioactive mevalonic acid of specific activity 0·05 μc/μmole was added to the flasks to give about 3 μmoles/g tissue.

Preparation and incubation of tissues. Rats and guinea-pigs were killed by a blow on the head, rabbits by the intravenous injection of 20 ml. of air. Blood was taken from the heart before death. After the death of the animal the liver was removed, weighed, and immersed in ice-cold buffer solution. Slices of the liver were cut by hand, using a long razor blade and a Perspex guide. About 1 g of slices was used from rats and about 2 g from rabbits and guinea-pigs. All incubations were begun within 20 min of the death of the animal. Skin for incubation was cut in thin strips about 5 mm wide from the back of the animal after removal of the fur. All subcutaneous fat was removed, but no attempt was made to separate the dermis from the epidermis.

All incubations were done in duplicate at 37° C for 3 hr. At the end of this time an equal volume of alcohol and 2 ml. of saturated NaOH/g tissue were added to kill the tissue. The flasks were then left throughout the night on a hot water-bath to hydrolyse the glycerides and cholesteryl esters.

Extraction of cholesterol and fatty acids. Cholesterol as digitonide, and fatty acids as soaps, were isolated from the contents of the flasks after incubation, essentially in the manner described by Gould, Taylor, Hagerman, Warner & Campbell (1953). About 10 mg cholesterol digitonide/g tissue was obtained from the livers and about 5 mg/g from the skins. The yield of soaps was about 25 mg/g liver.

Oxygen consumption. The q_{O_2} of the liver was measured in duplicate in slices taken from the stock and incubated in 4 ml. of the buffer, using a conventional Warburg apparatus. The gas phase was air; equilibration time 20 min. The results were expressed in μl. O_2 consumed/hr/mg wet tissue.

Estimations of cholesterol. The total cholesterol in fresh pieces of tissue and in plasma was determined by a modification of the method of Abell, Levy, Brodie & Kendall (1952). All estimations were done in duplicate.

Measurement of radioactivity. Radioactivity was measured with an end-window counter (EHM 2/S, G.E.C. Research Laboratories) having a thin mica window (1·7 mg/cm^2). The statistical counting error was less than ±3%. A standard ^{14}C source of known specific activity was measured at the beginning and end of each series of determinations. All counting rates were corrected to the values at infinite thickness by means of a standard self-absorption curve.

Determination of amount synthesized. The amount of cholesterol synthesized from added precursor by 1 g of tissue during the incubation was calculated as follows:

Let C = counts/min at infinite thickness of the sample;
P = cholesterol concentration in the tissue (μg/g);
S = specific activity of precursor used (μc/μmole); and
R = counting rate of μc/g standard.

Then the specific activity of the digitonide $= \frac{C}{R}$ μc/g.

$$\text{Total yield of digitonide} = P \times \frac{\text{mol. wt. digitonide}}{\text{mol. wt. cholesterol}}\ \mu\text{g}$$

$$= \frac{PC}{R} \times \frac{1602 \times 10^{-6}}{387}\ \mu\text{c}$$

= no. of μc of precursor appearing as digitonide

$$= \frac{PC}{RS} \times \frac{1602 \times 10^{-6}}{387}\ \mu\text{moles.}$$

Of the carbon atoms of cholesterol twelve are derived from the carboxyl group of acetate; therefore

$$\mu\text{moles of cholesterol formed with [1-}^{14}\text{C]-acetate as precursor} = \frac{PC}{RS} \times \frac{1602 \times 10^{-6}}{387 \times 12}$$

$$= \frac{PC}{RS} \times 1{\cdot}34 \times 10^{-4}\ \mu\text{g.}$$

Similarly, of the carbon atoms of cholesterol five are derived from C-2 of mevalonic acid; therefore

μmoles of cholesterol formed with [2-^{14}C]-mevalonic acid as precursor

$$= \frac{PC}{RS} \times \frac{1602 \times 10^{-6}}{387 \times 5}$$

$$= \frac{PC}{RS} \times 3{\cdot}21 \times 10^{-4}\ \mu\text{g.}$$

RESULTS

Metabolism of rat liver

Measurements were made on liver slices from thyroidectomized and thyroxine-treated rats, and from untreated rats kept in the same conditions and given the same diet as the treated animals. The average q_{O_2} of liver from normal rats was 0·827 ± 0·030 μl. O$_2$/mg wet wt./hr. After destruction of the thyroid there was a slight fall (Fig. 1*a*), but the difference from the control values was not significant. After injections of thyroxine varying from 10 to 500 μg/day for 10 days the q_{O_2} increased, the increase being apparent after injections of 10 μg/day. With larger doses there was a tendency for the q_{O_2} to reach higher

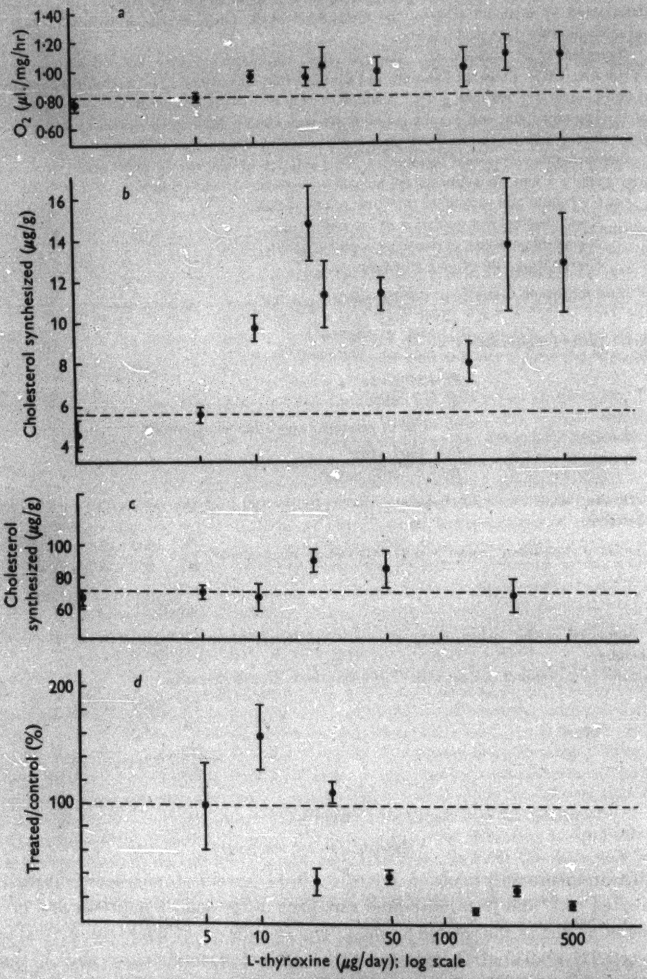

Fig. 1. Metabolism of liver slices from rats after destruction of the thyroid or injections of thyroxine, measured by (*a*) q_{O_2}; (*b*) rate of synthesis of cholesterol from acetate; (*c*) rate of synthesis of cholesterol from mevalonic acid; (*d*) rate of synthesis of fatty acid from acetate. Control rats are assumed to secrete 5 µg endogenous hormone/day, hypothyroid rats 1 µg/day. Vertical lines show S.E. of means.

values, but this was not very marked. In Fig. 1 we have assumed that the untreated rats secrete 5 μg endogenous thyroxine per day (Dempsey & Astwood, 1943); for the hypothyroid rats we have assumed an arbitrary value of 1 μg per day.

The average rate of synthesis of cholesterol from labelled acetate in the normal liver slices was 5·5 ± 0·4 μg/g of liver during the 3 hr period of incubation (Fig. 1*b*). After removal of the thyroid the rate fell to about 82 % of the control value. After injections of thyroxine it increased to between two and three times the control value. There was a definite increase in the slices taken from rats given 10 μg/day, but the increase was more marked after the higher doses.

The rate of synthesis of cholesterol from labelled mevalonic acid was much greater than that from labelled acetate. In the normal slices the rate of synthesis from mevalonic acid was 72 ± 5 μg/g during the period of incubation (Fig. 1*c*). In the absence of the thyroid there was a slight fall, but this was not statistically significant. After injections of thyroxine there was a slight increase with doses of 20 and 50 μg/day, but the effect was much smaller than that seen when acetate was the precursor.

In the experiments on fatty acid synthesis from acetate the results were so variable that it is impossible to draw any definite conclusions as to the effect of destruction of the thyroid or of small doses of thyroxine. However, with doses of 50 μg/day or more there was a marked reduction in the rate of synthesis (Fig. 1*d*). With the largest doses the rate fell to about 20 % of the control value.

Metabolism of rabbit liver

In rabbits the rate of synthesis of cholesterol by the liver varied widely from day to day. Accordingly, for each experiment a pair of rabbits was kept in the same cage and given the same diet, one rabbit receiving the treatment and the other acting as a control. They were then killed on the same day and the values from the treated animal expressed as percentages of the values from the untreated one. In Fig. 2 the results of all comparisons at each dose have been averaged. As with rats, we have assumed that the normal rate of secretion of endogenous thyroxine is 5 μg/day; we have also assumed the same value of 1 μg/day for the hypothyroid rabbits.

In the liver slices from the untreated rabbits the average q_{O_2} was 0·456 ± 0·018 μl. O_2/mg wet wt./hr. In the absence of the thyroid the q_{O_2} fell to 75 % of the normal value and after injections of thyroxine it rose above the normal value (Fig. 2*a*). When the daily dose was 10 μg there was an increase in the q_{O_2} of about 5 %; with larger doses it rose to 60 % above the normal.

In the normal liver slices the rate of synthesis of cholesterol from labelled acetate was 2·9 ± 0·4 μg/g during the incubation period. After destruction of the thyroid there was a significant reduction (Fig. 2*b*). After daily injections of 10 μg of thyroxine the rate did not differ significantly from the normal. With larger doses there was a reduction, the rate falling to 28% of the control value when the daily dose was 250 μg.

The rate of synthesis from mevalonic acid (14 ± 1·4 μg/g/3 hr incubation, in the normal slices) was greater than that from acetate. In the hypothyroid animals the rate did not differ significantly from the control value (Fig. 2*c*).

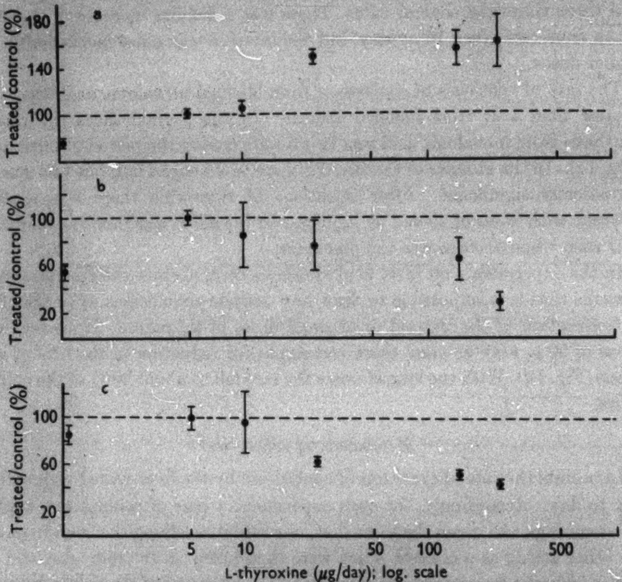

Fig. 2. Metabolism of liver slices from rabbits after destruction of the thyroid or injections of thyroxine, measured by (*a*) q_{O_2}; (*b*) rate of synthesis of cholesterol from acetate; (*c*) rate of synthesis of cholesterol from mevalonic acid. Thyroxine secreted by normal and hypothyroid rabbits assumed to be the same as in rats. Vertical lines show S.E. of means.

After injections of thyroxine the rate fell more or less in parallel with the rate of synthesis from acetate.

Fatty acid synthesis was measured in a few experiments. The rates of synthesis after destruction of the thyroid and after thyroxine treatment did not differ significantly from the control values. However, we cannot exclude the possibility that the treatments had some effect, since the variability among the untreated animals was very great.

Cholesterol synthesis in guinea-pig liver

In a few experiments the synthesis of cholesterol from acetate was measured in liver slices from normal and hypothyroid guinea-pigs. In the normal livers the rate of synthesis was 5·1 ± 0·1 μg/g liver during the 3 hr period of incubation and after thyroidectomy, 2·5 ± 0·1 μg/g/3 hr.

Synthesis of cholesterol by skin

Gould & Taylor (1950) have shown that skin synthesizes cholesterol from acetate *in vitro*. The synthesis of cholesterol from labelled acetate was therefore measured in slices of skin from normal, hypothyroid and thyroxine-treated rabbits, and from normal and hypothyroid guinea-pigs. The rate of synthesis was 0·38 ± 0·04 μg/g/3 hr in the normal rabbits and 0·52 ± 0·15 in the normal guinea-pigs. After destruction of the thyroid and after thyroxine treatment the rate did not differ significantly from the normal (Table 1).

TABLE 1. Synthesis of cholesterol from acetate by skin (μg/g/3 hr)

	Thyroidectomized	Controls	Thyroxine-treated
Rabbits	0·52 ± 0·15	0·38 ± 0·04	0·32 ± 0·03
Guinea-pigs	0·60 ± 0·09	0·52 ± 0·15	—

TABLE 2. The concentration of cholesterol in liver and plasma of rabbits and in the liver of rats (mg/100 g tissue)

		Thyroidectomized	Controls	Thyroxine-treated
Rabbits	Liver	300·5 ± 11·4	322·3 ± 3·1	355·4 ± 18·9
	Plasma	142·2 ± 17·6	73·5 ± 6·6	61·0 ± 6·8
Rats	Liver	249·1 ± 10·4	251·8 ± 7·5	288·8 ± 3·0

The rate of synthesis of cholesterol from labelled mevalonic acid was measured in skin slices from some of the normal rabbits. In contrast with the findings in liver, the rate was only a little greater (0·46 ± 0·07 μg/g) than the rate of synthesis from acetate. Since the skin slices were much thicker than the liver slices, this difference may possibly be explained by failure of the mevalonic acid to penetrate throughout the skin.

Plasma and liver cholesterol

The concentration of cholesterol was measured in the livers of the rats and rabbits and in the plasma of the rabbits (Table 2). In rabbits there was a rise in the plasma cholesterol after destruction of the thyroid and a fall after treatment with thyroxine; in the liver, changes in the opposite direction were observed. In rats similar changes were observed in the liver, but they were less marked than in rabbits.

Effect of addition of thyroxine and triiodothyroacetic acid in vitro

When L-thyroxine and L-triiodothyroacetic acid (TRIAC) were added at the beginning of the incubation to the flasks containing normal liver slices, there was no effect on cholesterol synthesis from labelled acetate or from labelled mevalonic acid (Table 3).

TABLE 3. Effect of addition *in vitro* of thyroxine and triiodothyroacetic acid (TRIAC) on synthesis of cholesterol by liver slices from rats and rabbits (μg cholesterol synthesized/g liver/3 hr incubation with acetate or mevalonic acid)

		Concn. (M)	Acetate as precursor	Mevalonic acid as precursor
Rats	Control	—	5·5 ± 0·37	72 ± 4·7
	Thyroxine	10^{-6}	5·1 ± 0·31	70 ± 2·5
Rabbits	Control	—	3·1 ± 0·43	12·7 ± 2·8
	Thyroxine	10^{-6}	3·0 ± 0·52	—
	Thyroxine	10^{-5}	3·3 ± 0·45	—
	Thyroxine	10^{-4}	3·3 ± 0·25	—
	TRIAC	10^{-6}	3·0 ± 0·31	—
	TRIAC	10^{-5}	2·7 ± 0·26	—
	TRIAC	10^{-4}	3·1 ± 0·27	12·5 ± 3·4

DISCUSSION

Synthesis of cholesterol and fatty acids by rat liver

The effects of thyroidectomy and thyroxine treatment on the synthesis of cholesterol from acetate *in vitro* are in agreement with previous work on rats *in vivo* (Rosenman *et al.* 1952; Byers, Rosenman, Friedman & Biggs, 1952). Rosenman *et al.* obtained their effects with very large doses of thyroid hormone (0·3 % of the diet as dried thyroid). In this work an effect was obtained when the rats were given 10 μg L-thyroxine/day. Since under these conditions the secretion of endogenous thyroid hormone is suppressed, the total amount of thyroxine received by the rat would be only about twice the normal rate of secretion from the thyroid, shown by Dempsey & Astwood (1943) to be about 5 μg/day. It is possible, therefore, that the effect we have observed *in vitro* occurs under physiological conditions.

The changes in the q_{O_2} were roughly in parallel with the changes in the synthesis of cholesterol from acetate (Fig. 1 *a*, *b*), an increase occurring in both measurements when the daily dose of thyroxine was 10 μg. This might suggest that the two effects are brought about by a single primary action of thyroxine. There is, for instance, some evidence (Du Toit, 1952) that thyroxine causes a rise in the Q_{O_2} by interfering with the normal mechanism whereby the energy derived from oxidation is used to form ATP. However, it is unlikely that the changes we have observed in cholesterol synthesis are due to this effect, since the synthesis of cholesterol from acetate is known to require ATP (Rabinowitz & Gurin, 1954). Any reduction in the synthesis of ATP would therefore be expected to reduce cholesterol synthesis. The inhibition of fatty acid synthesis

(Fig. 1*d*) could, on the other hand, be explained by this mechanism, though we have no evidence that the amount or availability of ATP was reduced in the livers from the treated animals. Since fatty acids (Seubert, Greull & Lynen, 1957; Lachance, Popják & de Waard, 1958) and cholesterol (Bucher & McGarrahan, 1956) are both synthesized in similar fractions of liver cells, the depression of fatty acid synthesis might be thought to be due to competition between fatty acids and cholesterol for a common precursor or co-factor. If so, free acetate is unlikely to be the precursor involved in this competition, since it was always present in excess.

In all these experiments, with both labelled acetate and labelled mevalonic acid, an excess of substrate was added to the medium. Under these conditions the slices made much more cholesterol from mevalonic acid than from acetate (Fig. 1*b*, *c*), even after maximum stimulation by thyroxine. The simplest explanation for this is that the enzymes converting mevalonic acid to cholesterol have a greater capacity than those converting acetate to mevalonic acid. On this hypothesis it is possible to explain the observation that alterations in thyroid function affect the synthesis of cholesterol from acetate but not from mevalonic acid, by supposing that the thyroid influences the rate of conversion of acetate to mevalonic acid but has little or no influence on the subsequent steps in the formation of cholesterol. As long as the capacity of the enzymes concerned in the steps beyond mevalonic acid is not exceeded, the rate of synthesis of cholesterol from acetate would be determined by the rate at which mevalonic acid is formed.

This agrees with the conclusions reached by other workers. It has been shown that lethal doses of X-irradiation (Gould & Popják, 1957) and injections of the synthetic detergent Triton WR-1339 (Bucher, 1959) increase the rate of synthesis of cholesterol from acetate, but have only a small effect on synthesis from mevalonic acid. Gould & Popják (1957) have also shown that a diet rich in cholesterol has little effect on cholesterol synthesis from mevalonic acid, although it is well known that cholesterol feeding markedly inhibits cholesterol synthesis from acetate.

Taken together, all these observations suggest that cholesterol synthesis in the living animal is regulated by control of the rate of synthesis of mevalonic acid. Possibly all the factors mentioned above—thyroid hormone, X-irradiation, Triton and cholesterol feeding—act upon the same link in the biosynthetic chain. However, this is not necessarily the case since there are several steps between acetate and mevalonic acid.

Synthesis of cholesterol by rabbit liver

In the experiments on rabbits, destruction of the thyroid had the same effect on cholesterol synthesis as in rats. The effect of injections of thyroxine, however, was quite different, synthesis from acetate and mevalonic acid being significantly reduced with large doses. The inhibitory action of thyroxine could be explained by interference with the formation of ATP, since ATP is required for the synthesis of cholesterol from acetate (Rabinowitz & Gurin, 1954) and from mevalonic acid (Popják, Gosselin & Gore, 1958). However, it is difficult to see why rats should not respond to thyroxine in the same way as rabbits. One way of explaining the difference between rats and rabbits is to suppose that thyroxine at low concentrations stimulates cholesterol synthesis from acetate, but that at high concentrations the inhibitory action predominates. Rats possess a very efficient mechanism for eliminating an excess of exogenous thyroxine (Gross & Leblond, 1950; Myant, 1957), whereas rabbits do not (Brown-Grant & Gibson, 1955). Concentrations of thyroxine high enough to cause depression of cholesterol synthesis may, therefore, be more difficult to achieve in rats than in rabbits. It is, perhaps, relevant that the largest doses caused signs of thyroxine-poisoning in rabbits but not in rats.

Against this explanation it may be argued that a stimulating action should have been observed in rabbits given small doses of thyroxine. However, the results obtained with injections of 10 μg/day were so variable that a small increase in the rate of synthesis from acetate cannot be excluded.

Effect of additions in vitro

Thyroxine added *in vitro* to normal liver slices had no apparent effect on cholesterol synthesis (Table 3). This is not surprising, since other workers have failed to observe an increase in the O_2 consumption of tissue slices when thyroxine is added *in vitro*.

TRIAC is probably a normal product of the metabolism of thyroxine in the tissues, though its presence has only been demonstrated by chromatographic methods (Roche, Michel, Jouan & Wolf, 1955). There is some evidence that, unlike thyroxine, it increases the O_2 consumption of kidney slices when added *in vitro* without a preliminary period of incubation with the tissue (Thibault & Pitt-Rivers, 1955). This has led to the suggestion that TRIAC is the biologically active form of the thyroid hormone. However, our failure to find any effect when TRIAC was added *in vitro* gives no support to this view.

Plasma concentration of cholesterol

The rise in the plasma cholesterol in the hypothyroid rabbits and the fall after thyroxine treatment (Table 2) are in agreement with earlier work on animals, and with the well known clinical observation that the plasma cholesterol is raised in myxoedema and tends to be below the normal in thyrotoxicosis. Clearly, these changes cannot be explained by the effects of absence of the thyroid and thyroxine treatment observed by us in rats and reported by others. There is some evidence that the rate of conversion of cholesterol to bile acids is reduced after destruction of the thyroid and increased after treatment with thyroxine (Thompson & Vars, 1954). This, rather than the change in the rate of synthesis of cholesterol, may explain the influence of the thyroid on the plasma cholesterol.

SUMMARY

1. The rate of synthesis of cholesterol from acetate and from mevalonic acid was measured *in vitro* in slices of liver and skin taken from rats and rabbits after destruction of the thyroid or injections of thyroxine.

2. In liver slices the rate of synthesis from mevalonic acid was much greater than the rate of synthesis from acetate.

3. In hypothyroid rats and rabbits there was a reduction in the rate of synthesis from acetate, but not from mevalonic acid. In guinea-pigs destruction of the thyroid also caused a reduction in the rate of synthesis from acetate.

4. In rats injections of thyroxine caused an increase in the rate of synthesis from acetate but not from mevalonic acid. In rabbits there was a depression of synthesis from acetate and mevalonic acid after doses of 150 μg or more of thyroxine.

5. The results in rats suggest that the thyroid influences the biosynthesis of cholesterol by increasing the rate of conversion of acetate to mevalonic acid. To explain the difference between rats and rabbits it is suggested that thyroxine stimulates cholesterol synthesis at low concentrations and inhibits it at high concentrations. In rats concentrations of thyroxine high enough to cause inhibition are not reached, because of the presence of an efficient mechanism for excreting thyroxine.

6. In liver slices from rats given large doses of thyroxine fatty acid synthesis was depressed.

7. Skin synthesized cholesterol from acetate and from mevalonic acid. There was no evidence that the rate of synthesis was influenced by destruction of the thyroid or injections of thyroxine.

We wish to thank Mr F. Gaebler for carrying out the cholesterol estimations, Mr L. Tout for looking after the animals, and Dr A. G. E. Pearse for reporting on the pituitaries. We are greatly indebted to Dr G. Popják for the ^{14}C-mevalonic acid, which was part of a batch prepared by Dr Rita Cornforth.

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THE EFFECT OF SINUSOIDAL STRETCHING UPON THE ACTIVITY OF STRETCH RECEPTORS IN VOLUNTARY MUSCLE AND THEIR REFLEX RESPONSES

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Voluntary muscle in most human subjects shows a periodicity in the discharge of groups of motor units, at a frequency about 9–10 c/s. These periodic 'bursts' of action potentials are accompanied by a corresponding mechanical oscillation. Under normal conditions this rhythmic activity is responsible for only a small fraction of the total tension being exerted by a muscle and, as shown by Lippold, Redfearn & Vučo (1957), produces a tremor which can be regarded as physiological. We argued that the effect was peripherally determined by self-oscillation in the stretch-reflex servo-loop, since the frequency of oscillation could be altered by cooling or stretching the muscle. The abolition of the rhythm by de-afferentation and by ischaemia of the muscle provides additional evidence for this hypothesis.

These facts do not, however, rule out the possibility of a central contributory factor. Continuous medullary stimulation is known to produce rhythmical activity in motoneurones at about 10 c/s independently of the frequency of stimulation (Bernhard, Skoglund & Therman, 1947). The 10 c/s precentral cortical rhythm might also contribute to such a central driving tendency, although Lindqvist (1941) has shown that the slowing of the alpha rhythm as a result of overbreathing is not accompanied by any change in the frequency of tremor.

The influence of any such central pace-maker upon the stretch reflex should be revealed by a study of the relationship between input to and output from the reflex centre. If there were a central rhythmical drive, then this relationship would be expected to vary in time with this rhythm.

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In the present experiments the time relations and amplitudes of response in muscle stretch receptors and those of the resulting reflex discharges have been measured during sinusoidal stretching of the tibialis anterior muscle in the cat. A sinusoidal input was chosen for two reasons: first, it is simple to define its parameters accurately and these can easily be controlled, and secondly the natural oscillations of tremor are quite close approximations to sine waves.

METHODS

Animal technique. Cats between 1·9 and 4 kg in weight and one dog were either lightly anaesthetized with veterinary Nembutal 30 mg/kg (sodium pentobarbital, Abbott Laboratories), given intraperitoneally, or were temporarily anaesthetized with ether before transection of the spinal cord at the level of C2 and destruction of the brain. Three cats were decerebrated at mid-brain level under temporary ether anaesthesia.

The tibialis anterior was prepared for recording by the method of Brown (1938). The nerve to tibialis anterior was exposed (by removal of the peroneal muscles), cut through as far away from the muscle as possible and placed for dissection and recording upon a black glass slide. Recordings were also made from the sartorius, the gracilis and the quadriceps extensor femoris muscles.

Recording methods. The electrical activity of the muscle was recorded from two wire electrodes inserted in the belly of the muscle, which was immersed in warmed paraffin. Conventional amplifiers and oscilloscopes enabled film or paper photographs to be taken. The nerve action potentials were recorded from wire electrodes piercing the nerve and led into a push–pull cathode follower. Dissection of the nerve under a microscope, distal to the point of recording, proceeded under paraffin and reduced progressively the number of active afferent fibres. In some experiments the dorsal roots S1, L7 and L6 were exposed and dissected until a single active fibre was obtained.

Electrical stimulation. The muscle was stimulated directly with square pulses of known frequency and of an amplitude and pulse width arranged to be (*a*) just supraliminal, or (*b*) supramaximal. Stimuli were given during a constant extension of the muscle.

Mechanical stimulator. The tendon of the muscle was attached to a mechanically driven slider, which oscillated sinusoidally along a guide rod at any predetermined frequency and amplitude. In certain experiments an RCA 5734 transducer was attached between the tendon and the slider to measure tension changes (Fig. 1). The slider carried the moving arm of a 20 kΩ wire-wound potentiometer, the resistance wire and former of which had been straightened and fixed to the cat myograph stand. This allowed the displacement of the mechanical system to be simultaneously displayed on the oscilloscope. The whole stimulator was arranged to slide along the line of oscillation so that the over-all stretch on the muscle could be adjusted during an experiment.

Photocell. It was necessary to verify that the phase lag between movement of the muscle belly and the mechanical stimulator was small compared with the time intervals which were of experimental interest. A photocell was used to measure the movement of a small marker stuck into the muscle surface at different points. The output from the photocell and the displacement potentiometer were then fed, after appropriate amplification, to the X and Y plates of an oscilloscope where a Lissajous's figure was produced (Fig. 2). At the frequencies and amplitudes employed in the experiments, the phase lag due to the visco-elastic properties of the muscle was in fact found to be negligible.

Measurement of records. Recordings were usually made on bromide paper travelling past the oscilloscope at 29·5 cm/sec. The phase angle of motor or sensory discharges was measured as follows: The mid point of the discharge, which in practically all cases corresponded with the point of maximum discharge (estimated visually or in some cases by measurement), was marked and projected on to the displacement sine wave. The interval between two successive peaks on the sine wave was measured and divided by the distance that this projection was in advance of the second peak. The resulting fraction multiplied by 360 gave the phase angle, θ, by which the discharge differed from the mechanical displacement. Thus, a phase angle of 0° indicated that the response was purely to displacement, 90° purely to velocity and 180° to acceleration (Fig. 3).

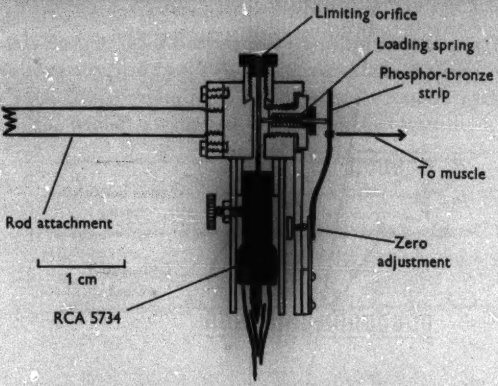

Fig. 1. Method of mounting the RCA 5734 transducer.

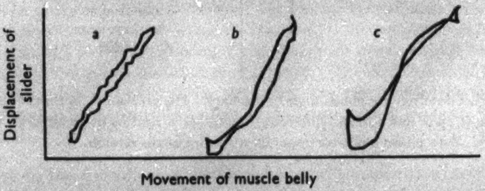

Fig. 2. Lissajous's figures produced by displacement of slider (X) and movement of muscle belly determined by a photoelectric cell (Y). (*a*) 5 c/s, (*b*) 10 c/s, (*c*) 20 c/s. Cat tibialis anterior muscle, normal blood and nerve supply.

RESULTS

General properties of the preparation

When the muscle was subjected to repetitive sinusoidal displacement, a contraction of its motor units occurred once in each cycle. The size of the recorded electrical discharges varied considerably and appeared to be sensitive to temperature changes and to the general condition of the experimental animal. If the muscle temperature fell too low, no response at all could be elicited upon stretching, although the response was quite normal in flexion and other reflexes. During slow stretches, or during spontaneous activity, the discharge of the motor units was usually in the form of groups of potentials, alternating with periods of quiescence, the group frequency being from 15 to 20 c/s.

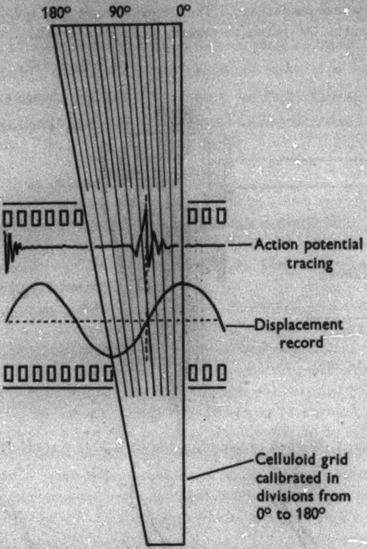
180°
90°
0°
Action potential tracing
Displacement record
Celluloid grid calibrated in divisions from 0° to 180°

Fig. 3. Method of determining phase angle of discharge. The grid is cut on thin celluloid sheet and by moving it up or down to suit different frequencies, a reading of phase angle can be arrived at directly. Zero phase angle corresponds with maximum stretch.

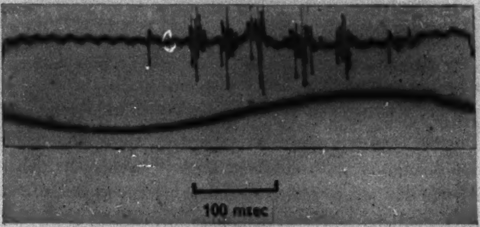

Fig. 4. Groups of muscle action potentials at 15–20 c/s during slow stretching of the tibialis anterior muscle. Intramuscular wire electrodes. Stretch at 1·5 c/s, amplitude 2·5 mm at the tendon. In this and subsequent records the displacement trace shows stretching as an upwards deflexion.

This is shown in Fig. 4 which is a record of muscle action potentials from the belly of the tibialis anterior being stretched sinusoidally at a peak velocity of 12 mm/sec. The reflex response consists of five separate bursts of motor unit activity.

The records from a typical experiment are illustrated in Fig. 5. The top recording (*a*) was obtained from two wires inserted into the muscle. It shows the motor response occurring once during each mechanical cycle; the displacement of the tendon is indicated by the sinusoidal tracing.

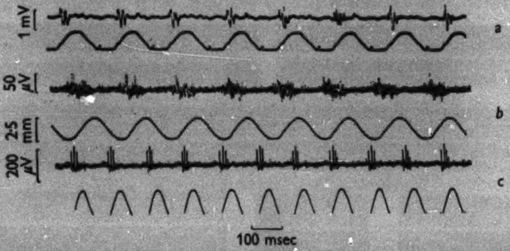

Fig. 5. Records of the time relations of motor and sensory discharges. (*a*) e.m.g. from muscle belly. (The mechanical trace is distorted but the mechanical stimulus was sinusoidal.) (*b*) Action potentials from whole cut nerve to muscle. (*c*) Action potentials from nerve after dissecting it down to 2 or 3 responding fibres. These were spindle endings because electrical stimulation of the muscle abolished their response. (The bottom half of the mechanical trace, corresponding with minimum stretching, was not included in the photograph.) Tibialis anterior; cat, anaesthetized with pentobarbitone sodium. A normal stretch response was present.

The nerve to the muscle was then isolated and the electrical record from it is shown at (*b*). The majority of the discharges were occurring once in each cycle, the most dense region being at a time when the muscle was being stretched. Dissection of the nerve reduced the number of active fibres until at (*c*) there were only two fibres remaining. These fired at the same phase in the cycle, namely around the time of fastest stretching.

The afferent discharges, recorded either in this way from a slip of muscle nerve or from the posterior roots, were found to be exceedingly sensitive to mechanical disturbances and even the driving motor (shaded-pole induction type) excited discharges at its rotational frequency if the vibration was transmitted to the operating table. The effect was particularly noticeable when the muscle was being stretched (cf. Echlin & Fessard, 1938).

The endings investigated were usually of the A_2, annulo-spiral, type, as was confirmed on liminal stimulation of the muscle electrically, when their resting discharge was interrupted for a short period, whereas supramaximal stimulation produced a short burst of impulses (Fig. 6). Tendon organs and A_1 endings were also observed but the data are not included in this paper.

Time relations of the sensory and motor discharges

The sensory discharges as well as the motor responses were recorded in the same animal, when the reflex arc was still intact, by splitting the dorsal roots in order to record from single stretch receptors. If the mid point of these grouped action potentials (motor as in Fig. 5*a*; sensory as in Fig. 6) was projected on to the mechanical record, it cut this near the maximum slope in each cycle.

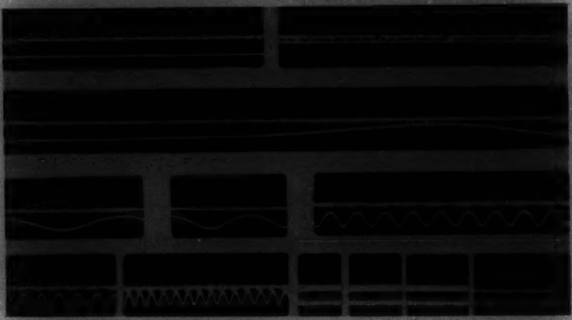

Fig. 6. The response to sinusoidal stretching of a single annulo-spiral (A_2) type stretch receptor in gastrocnemius of an anaesthetized cat. Top trace, action potentials recorded from single dorsal root fibre, ventral roots intact. Bottom trace, mechanical displacement of tendon (stretched upwards, 2·5 mm peak to peak). Top line, from left to right, static response after adaptation; four electrical stimuli of increasing voltage showing first a cessation of discharge, then a single response, then three responses to each stimulus. Second line, the response to gradually increasing stretch. Third line, response to sinusoidal stretching at various velocities. Bottom line the same (first two blocks) and the effect on the static response of twisting the pinna (third block, static response before eliciting pinna reflex, fourth, during twisting, fifth after reflex, time trace = 1/10 sec). The last block is a voltage calibration of 1 mV and time trace of 10 msec and 1/10 sec (for all records except pinna reflex).

Measurement of the phase angle showed the maximum discharge to be leading the displacement by approximately 90°, which meant that the muscle spindle response was greatest when velocity, rather than displacement, was maximal. In Fig. 5*a* the reflex motor discharges were retarded slightly, because of the delay in the reflex arc (6 msec in this experiment).

Inspection of Fig. 6 suggests a roughly constant discharge throughout the period of stretching, showing that the spindle responds to velocity rather than displacement. When either the motor or the sensory discharge showed a maximum in amplitude or frequency, this maximum, after allowing for conduction delay, occurred around the time of maximum velocity.

In the absence of any gamma motor activity (in the spinal preparations and in those with cut ventral roots or muscle nerves) the responses were very stable and the discharge patterns, firing frequencies and phase relations were constant for given velocities and frequencies of stretching the muscle. 5–10 min periods of repetitive stretching at frequencies up to 50 c/s seemed to have no detectable effect on the sensitivity of the spindle endings, although it should be noted that these were passive stretches with relatively small reflex effects, so that fatigue of the muscle as a whole was probably negligible. On the other hand, prolonged stretching beyond the normal physiological length of the muscle had the effect of increasing the resting discharge rate, presumably as a result of impairing the blood supply to the spindle (cf. Matthews, 1933).

The phase relations between muscle movement and the sensori-motor discharges as a function of the frequency of stimulation

The phase relations between the mechanical stimulus and the sensory discharge of stretch receptors were investigated at various amplitudes and frequencies of stretching. Fig. 6 illustrates an A_2 ending, as recorded from a single dorsal root fibre, responding at different frequencies of sinusoidal displacement.

After initially distinguishing the type of ending (by stimulating the muscle electrically) it was subjected to a slow stretch. During the lengthening of the muscle by the mechanical stimulator the ending fired repetitively, at a rate roughly proportional to the stretching velocity. The sinusoidal frequency was progressively increased, resulting in shorter discharges which were at higher frequencies, until stretching at 45 c/s produced only two discharges per cycle, separated by about 2 msec.

The muscle spindle at a constant extension was displacement-sensitive, i.e. it responded to a given extension of the muscle with a steady discharge. At very low velocities of stretch this was still true, but as velocity was increased, the mid point of the discharge (and also its instant of peak discharge rate) shifted to the left and instead of corresponding with the peak of the sine curve, appeared during the rising phase. This change from displacement-sensitivity to velocity-sensitivity was usually rapid and in most experiments occurred between stretching velocities of 0·1 to 0·5 cm/sec. When the sinusoidal frequency was reduced again, the stretch receptor became once more displacement-responsive without any change in its sensitivity.

These changes occurred always in the case of A_2 endings although the sensitivity of the endings (i.e. the number and frequency of sensory discharges at given velocities of stimulation) varied in different preparations. The anaesthetic seemed to have little effect, the same findings being true also in the spinal and decerebrate preparations. A_2 endings behaved similarly in flexor and extensor muscles, although there were slight differences in the reflex responses (see p. 382).

At frequencies between 4 and 15 c/s there was approximately 90° of phase difference between the sensory discharge and the displacement record. Allowing for the reflex time (the interval between a sudden stretch of the muscle and the first deflexion in the electrical record), the same was true for the motor discharge. Thus, over the range of frequencies and amplitudes at which oscillation may occur under natural conditions, the sensory endings are responding to the velocity of stretching rather than to displacement or tension.

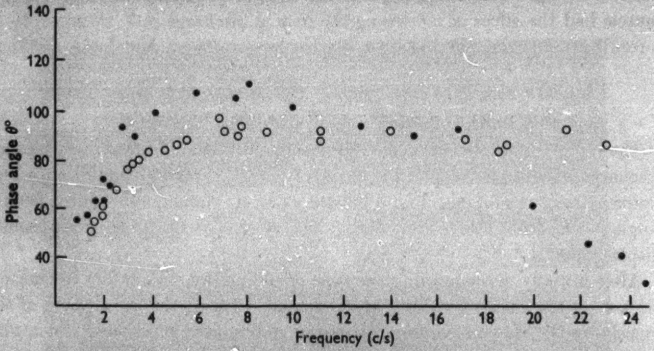

Fig. 7. The phase relations of (○) sensory discharge and (●) reflex motor action potentials at various frequencies of stretching. Amplitude of stretch = 2·5 mm. Allowance is made for a reflex time of 7 msec (in the case of the motor discharges only). The sensory discharge was obtained from the cut muscle nerve, whereas the motor action potentials were recorded before cutting the nerve. Spinal cat; tibialis anterior muscle.

The phase relationships between mechanical displacement and the discharges for an amplitude of 2·5 mm are shown in Fig. 7. At low frequencies the sensory discharge (reflex arc cut) became more and more proportional to the length of the muscle until, at zero frequency, the sensory endings were responding entirely to displacement. At frequencies up to about 15 c/s the curves for the sensory and the motor discharges follow each other closely, except that the motor discharge appears to be advanced more than the sensory discharge in phase.

At frequencies above 15 c/s the two curves diverge; the spindles continue to respond to the velocity of stretch while the motor discharge is progressively retarded. This effect is obvious on watching the oscilloscope screen as the frequency of operation is increased and is of such a magnitude that it cannot be explained on the basis of errors in measurement of the reflex time. Since the stretch receptor activity does not show this phase shift, the phenomenon can only be due to factors operative when the reflex arc is intact.

Effect of different amplitudes

If the amplitude of the stimulating oscillation is altered, the effects of velocity and frequency of stimulation can be separated. Fig. 8 shows the phase angle of motor discharges varying with mechanical stimulation frequency. The curves were obtained at two different amplitudes and hence the peak velocities varied. The curve produced at the greatest amplitude rises to 90° (α velocity) more rapidly, showing that the effect is the result of rate of stretch and not of frequency of stretching.

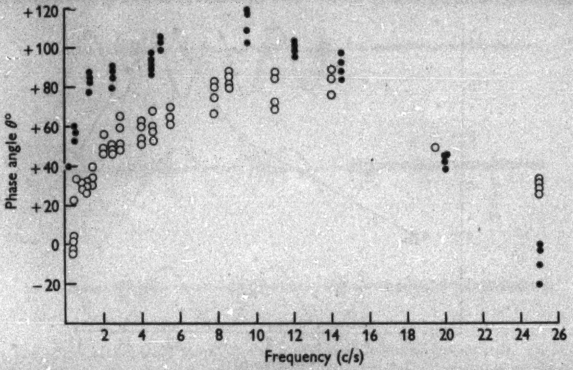

Fig. 8. The phase relations of reflex discharges at two different amplitudes of stretching and at varying frequency; ○, amplitude = 1·0 mm, ●, 2·5 mm; without allowance for reflex time.

Effect of reinforcement and inhibition

The effects of reinforcement and inhibition of the stretch reflex were studied by means of cutaneous stimulation of the ipsilateral and contralateral limbs. Reinforcement increased the magnitude of the motor and sensory discharges but these then began earlier in the cycle and ended later so that there was little net effect on the phase angle. Inhibition had a similar result; although the discharges were reduced in duration and amplitude, the phase relations remained approximately the same.

Effect of alteration of tension in the muscle

If the muscle was gradually lengthened during continuous sinusoidal stimulation, although the action potential bursts became larger there was no alteration in the phase of the cycle at which they were appearing. This appeared to rule out any variable lag in spindle response due to changes in the degree of mechanical coupling between spindles and the main muscle.

Relation between stretching frequency and the size of the reflex motor response

The magnitude of the motor response was measured roughly by adding together the amplitudes of the individual action potential spikes. This value varied with the frequency of mechanical stimulation, as is shown in Fig. 9. It can be seen that between 9 and 14 c/s there is a peak in the size of the integrated action potential. This is in keeping with the possibility that a certain degree of self-oscillation is taking place in the stretch reflex servo-loop, although it does not rule out a central augmentation at this frequency.

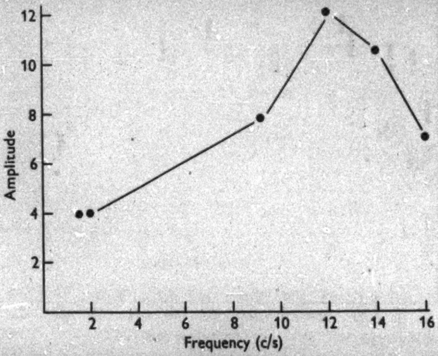

Fig. 9. The magnitude of the reflex discharge (arbitrary units, measured by adding spike heights in a burst) plotted against frequency. This shows a peak between 9 and 16 c/s, the frequency of spontaneous periodicity in the cat. Tibialis anterior; amplitude of stretch 2·5 mm; anaesthetic light pentobarbitone sodium.

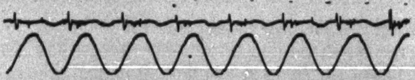

Fig. 10. Recording of motor discharge from rectus femoris. The response in this case shows a static component (α displacement) of small amplitude. Cat, anaesthetized with sodium pentobarbitone injected intraperitoneally (30 mg/kg body wt.).

Differences in response between flexor and extensor muscles

There was a distinct difference between the motor discharges from flexor and extensor muscles. Whereas in flexor muscles there was a single burst of motor activity with a peak approximately at its mid point, the discharge from extensor muscles showed an apparently separate, more prolonged burst of activity around the time of maximum displacement (Fig. 10). This presumably reflects a low-grade displacement response from some part of the population of stretch receptors but its source has not been clearly identified in the present series of experiments. This phenomenon was always to be seen in the electromyogram, but no differences in the responses of single spindles could be detected when recording from the dorsal roots.

The effect of abrupt interruption of the stretch

The mechanical stimulus could be abruptly stopped at or just before the point of maximum stretch. The effects of this upon the reflex discharge from the muscle stretch receptors varied with the pinna reflex, which, according to Granit, Job & Kaada (1952) causes activation of the gamma motoneurones.

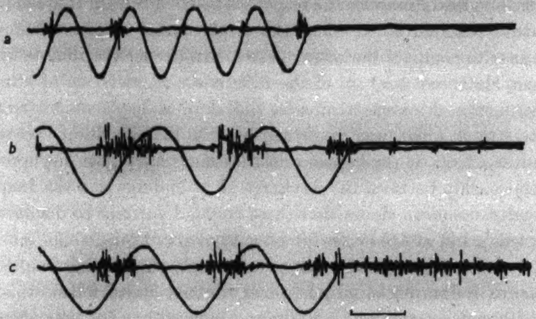

Fig. 11. The effect of suddenly stopping the mechanical stimulus (retouched). (*a*) With little gamma activity; (*b*) with moderate gamma activity; (*c*) with considerable gamma activity; time = 0·1 sec. Reflex motor discharges recorded from tibialis anterior, anaesthetized cat.

When there was little gamma activity, the burst of action potentials occurred at the instant of maximum velocity during the last cycle and electrical silence followed. With increased gamma activity there were several following bursts at approximately 80 msec intervals. Still more gamma activity resulted in a slowly decaying static component which commenced discharging after a short (10–30 msec) silent interval and continued for several seconds (Fig. 11 *a*, *b*, *c*).

DISCUSSION

During these experiments it was interesting to note the similarity between the discharges produced by passively stretching the muscle in the way described and those found previously in the normal human muscle. It is not possible to make an accurate comparison between the displacement of the spindles in the two cases because in the normal muscle active contractions were stretching the series elastic component and then exerting tension on the tendon; in these experiments the forces were being transmitted passively in the opposite sense, tendon movement stretching the muscle fibres. In terms of movement of the tendon it is probable that this was considerably greater in amplitude in the cat experiments.

It is certain, however, that the range of velocities of stretch employed covered those found in the normal muscle. In both cases it was clear that the stimulus to the spindles was velocity. This fact is not unexpected since the results of Matthews (1933) indicated that the response of muscle spindles had a velocity component, while Katz (1950) showed a static and a dynamic response to rapid stretches in the case of single spindles, although this was in frog muscle. From their records the largest ratio in frequency between the static and dynamic responses would appear to be about 1:4, but in our experiments on flexor muscles the responses above a certain frequency seemed to be entirely excited by velocity, the displacement response being absent. It is not easy to compare the rates of stretch in these experiments with those of Katz or Matthews because of the differences in methods of stimulation. Our experiments on extensor muscles did show a displacement response as far as the stretch reflex was concerned but the magnitude of this was quite small and its effects at the higher velocities must have been negligible.

The relationship between the discharge of A_2 endings and the frequency of mechanical stimulation shows that these respond entirely to displacement at zero frequency, but at 4–5 cycles (or velocity of about 0·1–0·5 cm/sec) they are excited purely by velocity and continue to be as the stimulating frequency rises.

The motor reflex discharges, however, do not quite follow this pattern because although responding similarly up to about 5 cycles they then become more advanced up to 10–15 c/s; at frequencies above this they become retarded again. It can therefore be concluded that the reflex arc does modify the response to muscle spindle stimulation as far as the time relations are concerned. The probable explanation is that the reflex servo-loop is resonating at 15 c/s. Let us suppose that at 15 c/s the system is resonant in the sense that the mechanical input and its reflex effects tend to reinforce each other. Then at slower frequencies one would expect the reflex motor discharge to occur sooner in the cycle of the mechanical input, while at frequencies higher than 15 c/s the reflex effects should occur later in the cycle than the instant corresponding with maximum reinforcement. This is in fact the effect which is observed.

This is in agreement with the hypothesis that oscillatory activity in the stretch reflex loop can occur and give rise to mechanical tremor. An effect similar to this would not be produced by a spontaneous periodicity of central origin, which would produce beats between its frequency and that of the mechanical stimulus. No such beats are observed.

When the muscle was stretched, the stretch reflex reinforced, or the pinna reflex elicited, there was practically no effect on the phase angle of the sensory or motor discharges. There was, however, an alteration, in each case, of the sensitivity of the sensory response, a finding which agrees with the recent work of Granit & Henatsch (1956) who, with a vibrator placed upon the skin over a muscle, stimulated spindles which became more sensitive when gamma efferent activity was simultaneously evoked. This lack of effect of stretch or gamma activity on the phase angle means probably that even when the muscle is at its shortest length and its spindles are relaxed, the degree of mechanical coupling between the spindles and the muscle as a whole is such that no detectable *additional* delay occurs between any displacement and the sensory response to it.

The most interesting feature of these results lies in the transition from displacement-sensitive to velocity-sensitive behaviour in the same receptor. This is clearly not due to a swamping of the displacement response by a larger velocity response, but to a progressive change in the response of the spindle. If a displacement component were still present during sinusoidal stimulation, there should at least be some activity at peak displacement, and the amount of this should be comparable with that present at zero or low frequency at the same tension. Instead of this we see a progressive shift in the phase angle of the response. Only in the case of extensor muscles does a displacement component persist in the reflex response, and this is clearly a separate phenomenon. Whether the transition described is due to changes in the electrical properties of the receptor membrane or in the physical state of the contents of the nuclear bag cannot at present be decided.

Katz (1950) has described static and dynamic components in the response of the frog spindle. He suggested that the underlying mechanism was concerned with the electrical properties of the sensory terminal. He inclined to the view that there were two separate electrical factors involved, namely, membrane capacity changes producing the dynamic element, and changes in membrane permeability being responsible for the static discharge. Such a hypothesis would entail a continuous response to a given stretch which would merely be modulated by dynamic changes, and not abolished. The positive receptor potential occurring as an 'off' effect on release from stretch cannot be responsible for the abolition of the displacement response during each cycle for two reasons: first, the duration of the positive potential seems too short; and secondly, the discharge often ceases while the muscle is still being stretched. Nevertheless, there may be other ways in which electrical changes in the sensory terminal might account for the results observed.

The nuclear bag must of necessity have viscosity and elasticity. Stretching it must therefore give rise to stress within its structure. Extension of a purely viscous fluid might be expected to give rise to internal stress proportional to the velocity, and extension of a purely elastic body would produce a force proportional to displacement. It is interesting to speculate about possible combinations of viscosity and elasticity, and about possible changes in these physical properties, which might account for our findings.

SUMMARY

1. Various muscles in the cat were stimulated mechanically with sinusoidal displacements of varying amplitudes and frequencies. The electrical activity of stretch receptors in the muscles and their reflex discharges were studied.

2. Comparison of the phase relationships between these discharges and muscle displacement at various frequencies showed that up to about 15 c/s both the sensory and the motor discharges (allowing for conduction delays) tended to be maximal at about the instant of greatest velocity of stretching. Above this frequency the spindles continue to respond maximally at this phase of the cycle but the reflex motor discharge occurs progressively later in the cycle. At no frequency were there any periodic changes in the size of the response.

3. At low velocities of stretching, below 0·1–0·5 cm/sec, muscle stretch receptors respond mainly to displacement. Above 3 cm/sec they respond entirely to velocity, in the tibialis anterior, although in extensor muscles there is a small displacement component in the reflex discharge.

4. The phase relations of the stretch receptor activity and the resulting motor discharge were not altered by increasing the tension on the muscle, alteration of the gamma efferent activity (as evoked reflexly) or by muscle temperature. These factors, however, did modify the sensitivity of the spindle response.

5. These results fail to show evidence of any central pace-maker which might be responsible for the rhythmical activity in normal voluntary muscle. On the other hand, they do demonstrate a resonant tendency on the part of the stretch reflex at the appropriate frequency.

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THE ACTION OF PHLORRHIZIN ON THE INTESTINAL TRANSFER OF GLUCOSE AND WATER *IN VITRO*

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The inhibitory effect of phlorrhizin on the intestinal absorption of glucose has long been known, and Fisher & Parsons (1950) showed that the inhibition could also be obtained *in vitro*. The effect of very small concentrations of phlorrhizin on the transport of glucose and of water has been studied by Jervis, Johnson, Sheff & Smyth (1956) and by Smyth & Taylor (1955*a*, 1957). The following experiments were undertaken to investigate further the inhibition by phlorrhizin of glucose and water transport and to find whether these inhibitions could be related to the effect of phlorrhizin on the metabolism of the intestine. As indices of metabolic activity oxygen consumption, glucose utilization and lactic acid production were chosen, and studies were made of the effects of various concentrations of phlorrhizin on these, and on the capacity of the intestine to transport water and glucose. The present paper is an amplification and extension of a preliminary report of Smyth & Taylor (1955*b*).

METHODS

The preparation used was the sac of everted small intestine of the rat described by Wilson & Wiseman (1954). This preparation offers a number of advantages, particularly in that oxygen consumption of the intestine can be studied in addition to other metabolic and functional activities. Wilson & Wiseman (1954) used sacs of 2–3 cm. In the experiments described below various lengths of sac were used. For measurement of oxygen consumption short sacs were found to be convenient, while in the transport experiments longer sacs as used by Smyth & Taylor (1958) were preferred. The technique of making the sacs was described by Wilson & Wiseman (1954). In describing the results the term 'serosal fluid' refers to the fluid in contact with the serosal surface, i.e. the fluid inside the sac, while 'mucosal fluid' is the fluid in which the sac is suspended. In the transport experiments the intestine was suspended in a Krebs-Ringer bicarbonate saline (Krebs & Henseleit, 1932). It is not, however, possible to measure oxygen consumption under these conditions and therefore in the experiments dealing with oxygen consumption the sacs were suspended in Krebs–Ringer phosphate saline (Krebs, 1933).

Study of respiration

Sacs of jejunum 2–3 cm long were used, filled with oxygenated Krebs'–Ringer phosphate solution. The sacs were suspended in 3 ml. of the same solution with a glucose concentration of 500 mg/100 ml., and the rate of respiration determined manometrically by the standard Warburg technique. After an equilibration period of 10 min the rate of oxygen uptake was measured for 30 min.

Phlorrhizin was then added from the side arm to give the required concentration and the respiration measured for another 60 min. In expressing the result the respiratory rate in the presence of phlorrhizin was compared with the rate during the initial period before adding the phlorrhizin. Control experiments were also carried out in which a total period of 90 min was used without any inhibitor present. In these control experiments the respiratory rate for the last 60 min was about 5% less than for the first 30 min and allowance was made for this in calculating the effect of various concentrations of phlorrhizin on the rate of respiration. In addition to measuring the effect of phlorrhizin, a number of determinations were made of the absolute rate of oxygen consumption of sacs. In fifteen experiments the Q_{O_2} expressed in the conventional way was $10{\cdot}8 \pm 1{\cdot}4$ µl. O_2/mg dry weight intestine/hr. In Fig. 2 this rate may therefore be taken as 100%. The figures in Fig. 2 can be used for approximate comparison of oxygen consumption with the other activities described, which are expressed per milligram wet weight. For this purpose the figure of 3·2 given by Fisher & Parsons (1953) for the wet weight: dry weight ratio can be used. It should be remembered, however, that these measurements are not strictly comparable because (1) the oxygen consumption was determined in phosphate saline, whereas the other changes were determined in bicarbonate saline, which in general enhances the activity of the intestine, and (2) in everted sacs part of the tissue under and near the ligatures is damaged or is less favourably oxygenated, and in the short sacs, where oxygen consumption was measured, the damaged part of the tissues forms a higher fraction of the total than it does in the larger sacs.

Study of transport and carbohydrate metabolism

Bicarbonate saline containing 500 mg glucose/100 ml. was used. Sacs of intestine 30 cm long were made from the jejunum. The sacs were filled with 3 ml. saline and were suspended in 15 ml. saline in 100 ml. conical flasks. The flasks used are shown in Fig. 1. The ligatures closing each end of the sacs were passed over the glass hook and this greatly facilitated removal of the sac at the end of the experiment. The flasks were shaken for 60 min at a rate of 80 c/min at 38° C, with a gas phase of 5% CO_2 and 95% O_2.

In the transport experiments it was essential to obtain samples of mucosal and serosal fluid separately for determination of the volumes and the concentration of glucose. In the metabolic experiments, on the other hand, the object was to determine the total amount of glucose used and also the lactic acid formed, and this could be done more accurately by pooling the mucosal and serosal fluids and adding to them an extract of the intestine. The details of the two types of experiments are as follows:

Transport experiments. A sac of everted intestine was weighed (W_1), filled with saline and weighed again (W_2). The sac was then suspended in a measured volume (15 ml.) of saline in the conical flask, gassed and shaken in the Warburg bath. At the end of this period the sac was removed, blotted with filter paper and weighed (W_3). The sac was then opened, the fluid drained out into a boiling-tube and the sac weighed again (W_4). Samples of the final serosal and final mucosal fluids were taken for analysis.

The various mucosal and serosal changes were calculated from W_1, W_2, W_3, W_4, and the initial and final glucose concentrations, as follows: The initial mucosal volume was always 15 ml., and was pipetted into the flask. The initial serosal volume was obtained from $W_2 - W_1$. The final serosal volume was obtained from $W_3 - W_4$. The difference between the initial and final serosal volumes gave the serosal volume change. It was found that W_4 (the final weight of the empty sac) was always greater than W_1 (the initial weight of the empty sac), and $W_4 - W_1$ gave the increase in fluid content of the intestine during the experiment. The final mucosal volume was obtained by subtracting from 15 ml. the sum of the volume of fluid uptake and the serosal volume change. In making these calculations, allowance was made for the weight of the ligatures, which may be either wet or dry according to the stage of the experiment. Standard lengths of ligature were used, and the weights of wet and dry ligatures were estimated so that the necessary corrections could be made. The initial and final amounts of glucose in the mucosal and serosal fluids were obtained by multiplying the appropriate volume by the corresponding glucose concentration.

Metabolic experiments. The procedure was the same as in the transport experiments until the end of the period of shaking in the Warburg bath. The conical flask was then plunged into a boiling water-bath for 2 min. The intestine was then removed and ground with sand. The extract was added to the mucosal and serosal fluid and all the washings were added to make up to a fixed volume. Samples of this were taken for glucose and lactic acid estimation.

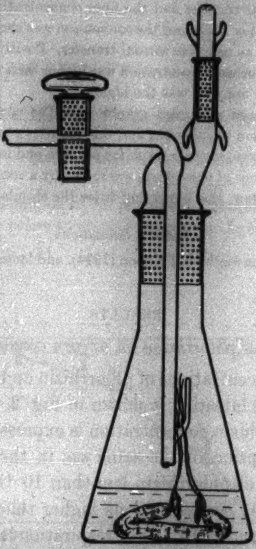

Fig. 1. Flask used for incubation of the sacs of everted intestine. The ligatures closing the sacs are looped over the glass hook and permit very easy removal of the sac at the end of the incubation period.

Methods of expressing results. The following terms are used in expressing the results:

Mucosal fluid transfer means the decrease in the volume of fluid on the mucosal side during the experiment;

Mucosal glucose transfer means the amount of glucose disappearing from the mucosal side;

Serosal fluid transfer is the increase in volume of fluid on the serosal side;

Serosal glucose transfer is the change in the amount of glucose in the serosal fluid;

Fluid uptake is the increase in the volume of the empty sac ($W_4 - W_1$) during the course of the experiment; and

Final concentration gradient is the difference between the final serosal glucose concentration and the final mucosal glucose concentration.

In the metabolic experiments, glucose utilization means the difference between the amount of glucose originally added and the amount of glucose found at the end of the experiment. Lactic acid production means the total increase in the amount of lactic acid in the whole system.

The amounts of glucose metabolized or transferred and the amount of water transferred have been expressed per milligram of initial wet weight of intestine. Calculation on a dry weight basis was not possible in the metabolic experiments, since the tissue had to be ground and extracted, and therefore all results have been given in terms of wet weight. The fluid transfer and fluid uptake are expressed in μl. The amounts of glucose and lactic acid are expressed in μg and the concentrations in mg/100 ml. In order to avoid confusion quantities have been considered positive as far as possible, e.g. decrease in mucosal volume of fluid is given as positive mucosal fluid transfer, and increase in serosal volume as positive serosal fluid transfer. The only cases where this was not possible are the serosal glucose transfer and the final concentration gradient. Since the serosal glucose may be either increased or decreased the convention was used of giving increases as positive serosal transfer and decreases as negative serosal transfer. Positive final concentration gradient implies that the final serosal glucose concentration was higher than the final mucosal, and negative gradient implies that the final mucosal was the higher.

The results have mainly been expressed graphically. The concentration of phlorrhizin was plotted on a logarithmic scale on the abscissa and the quantity studied on the ordinate, with the conventions indicated in the previous paragraph for positive and negative quantities. In all cases the average values in the absence of phlorrhizin are shown by a continuous horizontal line with an interrupted line above and below, this indicating twice the standard error of the mean values in the absence of phlorrhizin.

Chemical estimations

Glucose was estimated by the method of Nelson (1944), and lactic acid by the method of Elsden & Gibson (1953).

RESULTS

Effect of phlorrhizin on oxygen consumption

The effect of various concentrations of phlorrhizin on the rate of oxygen uptake by sacs of everted small intestine is shown in Fig. 2. The rate of oxygen consumption at each phlorrhizin concentration is expressed as a percentage of the rate of oxygen consumption of the same sac in the absence of phlorrhizin. At final concentrations of phlorrhizin less than 10^{-4} M there is little effect on oxygen consumption. At concentrations higher than this there is inhibition of oxygen consumption and at 10^{-3} M the respiration is depressed to about 60 % of the level in the absence of phlorrhizin. It is clear that phlorrhizin is not a powerful inhibitor of respiration and in concentrations of less than 10^{-4} M it has only a very small inhibitory action. Kalckar (1937) found that 2×10^{-3} M phlorrhizin caused only a small inhibition of respiration of kidney cortex slices. Fleischmann (1937) found a relatively small inhibition of oxygen consumption by kidney and liver slices with 2×10^{-3} M phlorrhizin, but a much greater inhibition in brain cortex, while Webb, Saunders & Thienes (1949) found 30 % depression of respiration by slices of rat heart muscle in the presence of 2×10^{-3} M phlorrhizin.

The effect of phlorrhizin on carbohydrate metabolism

The effects of various concentrations of phlorrhizin on glucose utilization and lactic acid production are shown in Figs. 3 and 4. A concentration of 10^{-5} M phlorrhizin produces an effect on glucose utilization which is possibly just significant. The average glucose utilization is 17·0 μg glucose/mg wet weight of tissue as compared with a value of 18·8 μg/mg wet weight in the

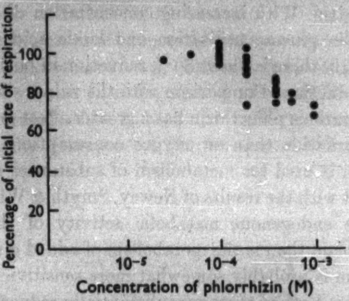

Fig. 2. Effect of phlorrhizin on the rate of oxygen consumption of sacs of everted intestine. The points show the rate of respiration in the presence of phlorrhizin expressed as a percentage of the rate in the same sac before addition of phlorrhizin. In all figures the abscissa is shown on log. scale.

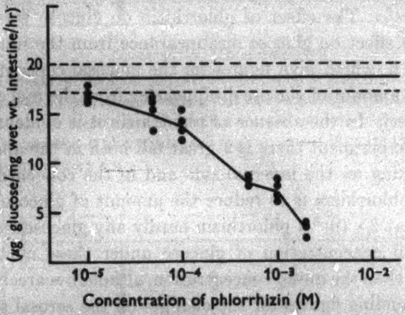

Fig. 3. Effect of phlorrhizin on the glucose utilization of sacs of everted intestine. The continuous horizontal line shows the mean value for glucose consumption in eight experiments in the absence of phlorrhizin, and the two dotted lines are drawn at a distance above and below this of 2 × S.E.M.

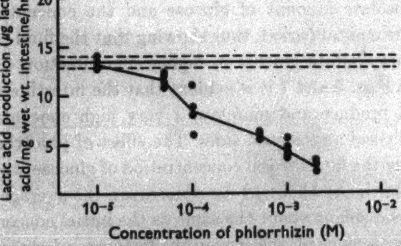

Fig. 4. Effect of phlorrhizin on the production of lactic acid by sacs of everted intestine. The continuous horizontal line shows the mean value of lactic acid production in eight experiments in the absence of phlorrhizin, and the two dotted lines are drawn at a distance above and below this of 2 × S.E.M.

absence of phlorrhizin. With increasing concentration of phlorrhizin there is a progressive fall in glucose utilization and lactic acid production, and at 2×10^{-3} M phlorrhizin there is about 80 % reduction in both glucose utilization and lactic acid production. Comparison with the values shown in Fig. 2 shows that at all concentrations phlorrhizin has a greater effect on glucose utilization and lactic acid formation than on oxygen consumption. This suggests that part of the oxygen is used for metabolism of substances other than glucose, and is in agreement with the results of Newey, Smyth & Whaler (1955). It also indicates that the endogenous metabolic activity of the intestine is less affected by phlorrhizin than is the metabolism of added glucose. While carbohydrate metabolism is probably somewhat more sensitive to phlorrhizin than oxygen consumption, both of these are substantially reduced only at the higher concentrations of phlorrhizin used.

Effect of phlorrhizin on glucose and water transfer

Glucose transfer. The effect of phlorrhizin on glucose transfer is shown in Figs. 5–9. The effect on glucose disappearance from the mucosal side is seen in Figs. 5 and 6, which give figures for the mucosal concentration of glucose and the actual amount of glucose disappearing from the mucosal side (mucosal glucose transfer). In the absence of phlorrhizin it is evident that during the period of the experiment there is a great fall both in the absolute amount of glucose remaining on the mucosal side and in the concentration of glucose. The effect of phlorrhizin is to reduce the amount of glucose passing into the intestine, and at 2×10^{-3} M phlorrhizin hardly any glucose leaves the mucosal fluid. The final concentration of glucose under these conditions is indeed slightly higher than the initial concentration, although scarcely significantly so.

The corresponding figures for the changes on the serosal side are shown in Figs. 7 and 8. These are more complicated than the mucosal changes, and in Fig. 8 positive values mean an increase in the absolute amount of glucose on the serosal side during the experimental period and negative values a decrease. In the absence of phlorrhizin it is seen that there is a very considerable increase both in the absolute amount of glucose and the concentration of glucose during the experimental period, thus showing that the fluid transferred to the serosal side must contain glucose in higher concentration than that present initially. From Figs. 5 and 7 it is evident that the intestine in the absence of phlorrhizin can produce and maintain a very high concentration difference between the mucosal and serosal sides. The effect of phlorrhizin is to cause a reduction both in the final serosal concentration of glucose, and in the absolute amount transferred. At about 10^{-5} M phlorrhizin the average final concentration on the serosal side is about the same as the initial concentration (Fig. 7). At higher concentrations of phlorrhizin the final serosal glucose concentration falls even further and the maximum effect is produced at about 5×10^{-4} M phlorrhizin. With concentrations of 10^{-3} M and 2×10^{-3} M phlorrhizin the fall in final serosal concentration of glucose is less pronounced and the probable reason for this will be discussed later. Phlorrhizin is also seen to reduce the absolute amount of glucose transferred, and at about 5×10^{-5} M phlorrhizin the serosal glucose transfer falls to zero. At higher concentrations of phlorrhizin glucose actually disappears from the serosal side, and this effect is

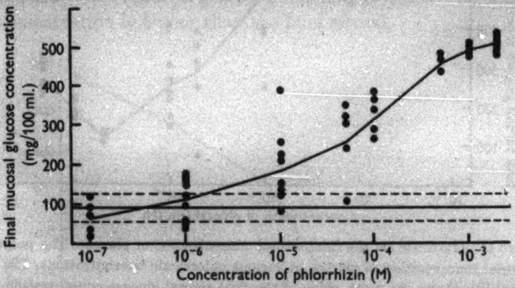

Fig. 5. Effect of phlorrhizin on the final mucosal concentration of glucose. The points show the final mucosal glucose concentration at various concentrations of phlorrhizin. The continuous horizontal line shows the mean value of the final mucosal glucose concentration in fourteen experiments in the absence of phlorrhizin, and the two dotted lines are drawn at a distance above and below this of 2 × S.E.M.

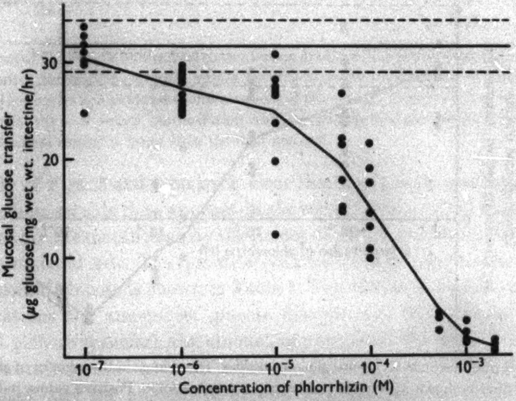

Fig. 6. Effect of phlorrhizin on mucosal glucose transfer. The points show the amount of glucose disappearing from the mucosal fluid at various phlorrhizin concentrations. The horizontal continuous line shows the mean value of mucosal glucose transport in fourteen experiments in the absence of phlorrhizin, and the two dotted lines are drawn at a distance above and below this of 2 × S.E.M.

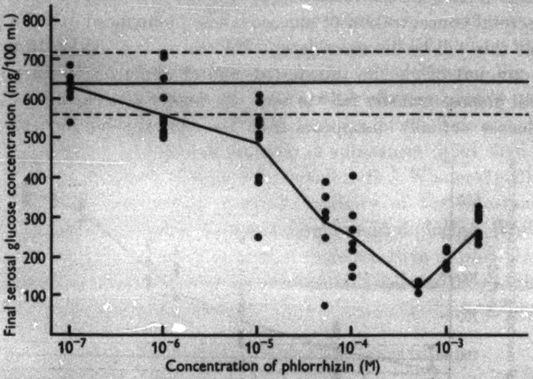

Fig. 7. Effect of phlorrhizin on the final serosal concentration of glucose. The points show the final serosal concentration of glucose at various phlorrhizin concentrations. The continuous horizontal line shows the mean value of the final serosal glucose concentration in fourteen experiments in the absence of phlorrhizin, and the two dotted lines are drawn at a distance above and below this of 2 × S.E.M.

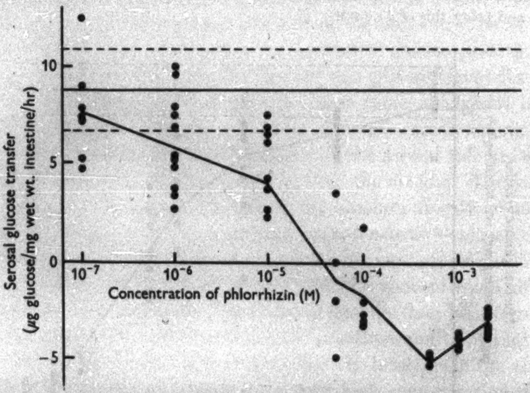

Fig. 8. Effect of phlorrhizin on serosal glucose transfer. The change in the amount of glucose in the serosal fluid is shown at various phlorrhizin concentrations. Positive values indicate an increase in the amount of serosal glucose and negative values a decrease. The continuous horizontal line shows the mean value for serosal glucose transfer in fourteen experiments in the absence of phlorrhizin; and the two dotted lines are drawn at a distance above and below this of 2 × S.E.M.

maximum at 5×10^{-4} M phlorrhizin. The effect of phlorrhizin on the mucosal and serosal concentrations of glucose is well seen in Fig. 9, which shows the final glucose gradient (difference between the final mucosal and final serosal glucose concentrations). In the absence of phlorrhizin a large positive gradient is found—the final serosal is higher than the final mucosal. As the phlorrhizin concentration is increased the final gradient is reduced and at high concentrations of phlorrhizin the final glucose gradient is reversed—the final mucosal glucose concentration is higher than the final serosal.

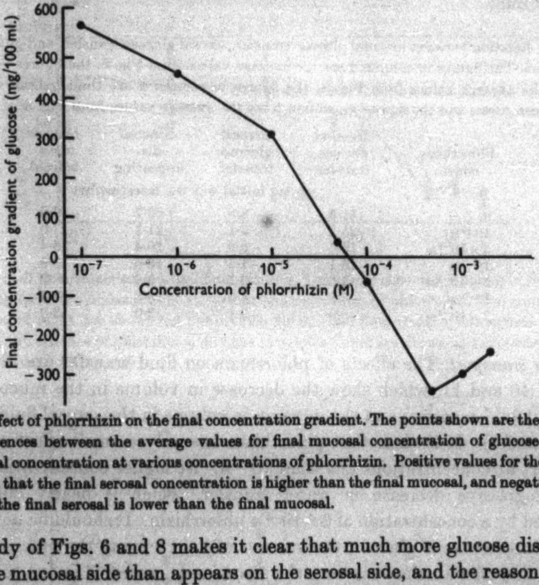

Fig. 9. Effect of phlorrhizin on the final concentration gradient. The points shown are the algebraic differences between the average values for final mucosal concentration of glucose and final serosal concentration at various concentrations of phlorrhizin. Positive values for the gradient mean that the final serosal concentration is higher than the final mucosal, and negative values that the final serosal is lower than the final mucosal.

A study of Figs. 6 and 8 makes it clear that much more glucose disappears from the mucosal side than appears on the serosal side, and the reason for this is presumably the metabolism by the tissues of part of the glucose disappearing from the mucosal side. The quantitative relationship between transport and metabolism of glucose is shown in Table 1. This shows for various phlorrhizin concentrations the amount of glucose disappearing from the mucosal side (mucosal glucose transfer), the amount appearing on the serosal side (serosal glucose transfer), the algebraic difference between these (total glucose disappearance), and the amount of glucose metabolized. It should be clearly understood that total glucose disappearance, as defined here, means disappearance from the mucosal and serosal fluids only, while glucose metabolized means disappearance of glucose from the whole system including the intestine. The figures given in Table 1 are the average values given in Figs. 3, 6 and 8.

It is seen that there is fairly good agreement between the total glucose disappearance in the transport experiments and the glucose metabolized in the metabolism experiments. In all cases the glucose disappearance is somewhat greater than the glucose metabolized. The discrepancy is probably accounted for by the glucose in the intestinal tissues which has disappeared from the surrounding fluids but has not been metabolized. It is also seen from Table 1 that at high concentrations of phlorrhizin the absolute amount of glucose leaving the serosal fluid is greater than the absolute amount leaving the mucosal fluid.

Table 1. Relation between mucosal glucose transfer, serosal glucose transfer, and glucose metabolized. The figures in column 2 are the average values from Fig. 6, the figures in column 3 are the average values from Fig. 8, the figures in column 4 are the algebraic differences between these, and the figures in column 5 are the average values from Fig. 3

Phlorrhizin concn.	Mucosal glucose transfer	Serosal glucose transfer	Glucose dis-appearing	Glucose meta-bolized
	μg/mg initial wet wt. intestine/hr)			
0	31·5	8·8	22·7	18·8
10^{-5} M	25·2	4·1	21·1	17·0
5×10^{-5} M	19·5	−0·9	20·4	15·4
10^{-4} M	15·1	−1·5	16·6	13·9
5×10^{-4} M	3·5	−5·7	9·2	8·2
10^{-3} M	−1·8	−4·0	5·8	7·3
2×10^{-3} M	0·8	−3·1	3·9	3·2

Water transport. The effects of phlorrhizin on fluid transfer are illustrated in Figs. 10 and 11, which show the decrease in volume in the mucosal fluid (mucosal fluid transfer) and the increase in volume in the serosal fluid (serosal fluid transfer). It is clear that phlorrhizin has only a small effect on water transport at concentrations less than 10^{-5} M. At higher concentrations there is a progressive decrease in water transfer, which is nearly completely abolished by a concentration of 2×10^{-3} M phlorrhizin. It should be noted that, just as in the case of glucose, the amount of fluid leaving the mucosal side is greater than the amount entering the serosal side, and this is due to the fact that the volume of fluid in the tissues increases. The increase in tissue fluid (fluid uptake) accounts for about one third of the fluid leaving the mucosal side. Fig. 12 shows the effect of phlorrhizin on fluid uptake, and it is seen that this is reduced at high concentrations of phlorrhizin.

Relation between glucose movement and water movement. The results presented show the profound effect of phlorrhizin on both glucose and water movement. Of great interest is the question whether these effects are parallel, in which case they might have a common cause, or whether glucose transfer is inhibited by phlorrhizin to a greater or lesser degree than water transfer. A study of Fig. 5 shows that in the presence of phlorrhizin the final mucosal concentration of glucose is higher than in its absence and this might be thought to show that phlorrhizin has more effect on glucose transfer than water transfer. Further consideration however will show that this conclusion is not warranted. The actual concentration of glucose in the fluid leaving the mucosal side can be calculated from the amount of glucose and the volume of fluid. If average values are taken it is found that in the absence of phlorrhizin 62·4 mg of glucose and 3·16 ml. of fluid leave the mucosal side, and the concentration of glucose in the fluid leaving is therefore 1·97 g/100 ml. Since the

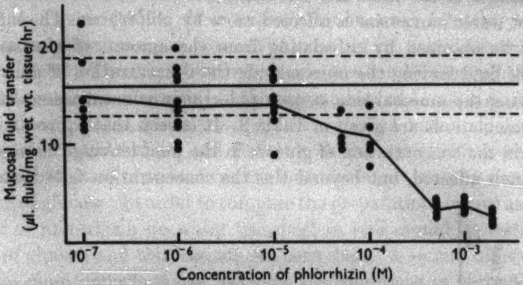

Fig. 10. Effect of phlorrhizin on mucosal fluid transfer. The points show the volumes of fluid disappearing from the mucosal side at various concentrations of phlorrhizin. The continuous horizontal straight line shows the mean value for mucosal fluid transfer in fourteen experiments in the absence of phlorrhizin, and the two dotted lines are drawn at a distance above and below this of 2 × S.E.M.

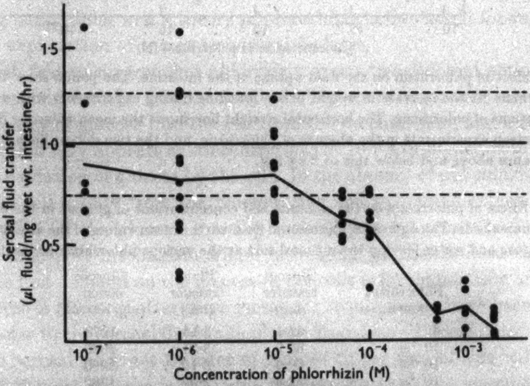

Fig. 11. Effect of phlorrhizin on serosal fluid transfer. The points show the increase in volume of serosal fluid at various concentrations of phlorrhizin. The horizontal straight line shows the mean value for serosal fluid transfer in fourteen experiments in the absence of phlorrhizin, and the two dotted lines are drawn above and below this at a distance of 2 × S.E.M.

initial mucosal concentration is 0·5 g/100 ml., the concentration of glucose in the fluid leaving the mucosal side is nearly four times that in the mucosal fluid. Whether the effect of phlorrhizin was to reduce the volume of fluid moved without altering the glucose concentration in it, or to reduce the concentration of glucose without altering the volume, in both cases the result would be to cause a higher concentration of glucose in the mucosal fluid at the end of the experiment than would be the case when no phlorrhizin was present. Thus, changes in the final mucosal concentration do not indicate whether glucose or water movement is affected more by phlorrhizin. The information can only be obtained by calculating from the amount of glucose and the amount of fluid leaving the mucosal side the concentration of glucose in the fluid leaving the mucosal side at each phlorrhizin concentration. The results of such calculations are given in Table 2. It is seen that up to about 10^{-5} M phlorrhizin the concentration of glucose in the fluid leaving the mucosal side is not much affected, but beyond this the concentration falls steadily, thus

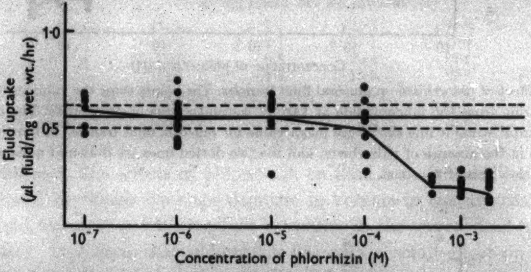

Fig. 12. Effect of phlorrhizin on the fluid uptake of the intestine. The points show the effect of phlorrhizin on the increase in weight of the intestine during experiments with various concentrations of phlorrhizin. The horizontal straight line shows the mean value for fluid uptake in fourteen experiments in the absence of phlorrhizin, and the two dotted lines are drawn at a distance above and below this of 2 × S.E.M.

TABLE 2. Effect of phlorrhizin on the amounts and concentrations of glucose in the fluid leaving the mucosal side. The figures for glucose and fluid are the mean values of the absolute amounts of glucose and water leaving the mucosal side at the various phlorrhizin concentrations

Phlorrhizin concn.	Glucose transfer (mg)	Fluid transfer (ml.)	Glucose concn. (mg/100 ml.)
0	62·4	3·16	1970
10^{-7} M	66·9	3·31	2020
10^{-6} M	60·6	3·10	1960
10^{-5} M	52·6	2·97	1780
5×10^{-5} M	41·1	2·42	1700
10^{-4} M	34·9	2·53	1380
5×10^{-4} M	7·1	0·72	985
10^{-3} M	3·68	0·76	452
2×10^{-3} M	1·84	0·52	354

indicating that at the higher phlorrhizin concentration the transfer of glucose is affected more than the transfer of fluid. The effects of phlorrhizin on mucosal glucose and water transport are thus not parallel. This is in agreement with the finding of Fisher & Parsons (1950) that 'in the presence of phlorrhizin water leaves the inner circuit more rapidly than glucose'.

DISCUSSION

The results obtained confirm and extend those of Fisher & Parsons (1950) and of Smyth & Taylor (1957). It is noteworthy that the inhibitory effect of phlorrhizin on water transport was not obtained in the experiments *in vivo* of Jervis *et al.* (1956). There are, however, two important differences between the experiments *in vitro* and *in vivo*. In the experiments of Jervis *et al.* (1956) the effects of phlorrhizin on water transport were studied with no glucose in the fluid in the intestinal lumen, and Fullerton & Parsons (1956) have recently shown that absorption of water from the intestine is increased by simultaneous absorption of glucose. In order to compare the preparations *in vivo* and *in vitro* the effect of phlorrhizin on water transport *in vivo* should be tested in the presence of glucose and this has not yet been done. A second difference, and probably a more important one, between the conditions *in vivo* and *in vitro* is that in conditions *in vivo* the supply of metabolic fuel to the intestinal cells does not depend on the presence of glucose in the intestinal lumen, but can presumably be maintained by the arterial blood supply, whereas in conditions *in vitro* the metabolic fuel must be obtained from the fluid in which the intestine is suspended. The significance of this difference is discussed in the following paragraphs, and a theory of phlorrhizin action is put forward which gives an explanation of the experimental findings.

We wish first to suggest that phlorrhizin exerts two different actions which are responsible for the results obtained. The more important action is to prevent entry of glucose into the cell from the mucosal side; and a secondary action, which only appears at concentrations of 10^{-3} M or higher, is to interfere with the oxidation of glucose by the cell. In the absence of phlorrhizin, glucose enters the cell mainly from the mucosal side and to a small extent from the serosal side. Of the glucose entering the cell part is metabolized and part enters a transport mechanism which ultimately transports it to the serosal fluid. The first demand on the glucose in the cells is for metabolism and only if any glucose is left over after these metabolic requirements have been satisfied will transfer to the serosal fluid take place. Since some glucose enters the cells from the serosal side, net transfer of glucose to the serosal side will only be apparent if more glucose is available for transfer than the amount entering the cells from the serosal side. In the presence of phlorrhizin the entry of glucose from the mucosal side is reduced. This means that less glucose is available for the combined demands of metabolism and transfer, and at a certain concentration of phlorrhizin the amount of glucose available for transfer is only sufficient to balance the amount metabolized from the serosal side. At such a concentration (about 10^{-5} M–2×10^{-5} M) there will be no increase in serosal glucose, there will still be some disappearance from the mucosal side, and there will be little effect on oxygen consumption, glucose disappearance or lactic acid production. As the phlorrhizin concentration is increased still less glucose enters from the mucosal side, and the glucose available in the cell is now insufficient for the metabolic activity of the cell. The result is that the serosal glucose level falls, and the glucose disappearance and lactic acid production and oxygen consumption also fall. A stage is reached when the concentration of glucose on the serosal side falls more rapidly than on the mucosal, and at this point the final concentration gradient is reversed. At 10^{-3} M phlorrhizin the second effect of phlorrhizin appears, inhibition of metabolism. The demand for glucose is reduced and less enters from the serosal side. This view is in keeping with the results given in Fig. 8 and Table 1. It is possible to test this hypothesis of phlorrhizin action by using labelled glucose on either the mucosal or serosal sides. This has in fact recently been done, and a preliminary report by Newey, Parsons & Smyth (1957) supports the hypothesis outlined here.

As an alternative possibility it might be suggested that the action of phlorrhizin is primarily on metabolism, and as a result energy is no longer available for transport. Taggart (1952) has in fact suggested that inhibition of transfer activities by phlorrhizin is only seen at concentrations which inhibit cellular oxidation. It is difficult to reconcile such a view with the finding that at higher phlorrhizin concentrations more glucose disappears from the serosal side than from the mucosal side (Table 1), unless it is also postulated that the disappearance of glucose from the serosal side is caused by metabolism of the muscle cells, and that this is unaffected by phlorrhizin which inhibits oxidation only in the mucosal cells. This seems a less satisfactory explanation of phlorrhizin action than the one suggested, that phlorrhizin prevents the entry of glucose from the mucosal side.

The findings as regards water transfer must now be considered. The relationship between glucose and water transfer is undoubtedly a complex one. Smyth & Taylor (1957) have suggested that at least two processes are concerned in glucose transport (apart from movement as lactic acid). These processes are (1) active transport of glucose which may take place against a glucose concentration gradient, and (2) passive transport of glucose in the water stream. It is now suggested that a somewhat similar reciprocity may exist in relation to water transport, i.e. part of the water movement may be due to an active process independent of glucose movement, while part may be due to glucose movement. The active movement of water, while not dependent on glucose transport, is, however, dependent on glucose metabolism. These views are in keeping with the well-known fact that water can be absorbed from the intestine *in vivo* without simultaneous absorption of glucose, while on the other hand as Fullerton & Parsons (1956) have shown, absorption of water from the intestine is increased by simultaneous glucose absorption. Phlorrhizin therefore interferes with water movement in the intestinal preparations *in vitro* (*a*) by disturbing glucose transport, and (*b*) by making glucose unavailable for cell metabolism. The present experiments do not throw any light on the nature of the active mechanisms for water transport, apart from showing that it is dependent on glucose metabolism. Hendley & Smyth (1957) have, however, recently shown that the intestinal preparation *in vitro* is capable of active movement of sodium and it seems possible that the active process responsible for water movement may be movement of sodium.

The suggestions made here apply to the site of phlorrhizin inhibition rather than to the manner in which it is brought about. In concentrations of 10^{-4} M and higher, phlorrhizin produces inhibition of various oxidative systems, as was shown for example by Shapiro (1947) and Lotspeich & Keller (1956). Effects of this nature are possibly responsible for the depression of metabolism described in these experiments with high phlorrhizin concentrations. A basis for the effect of the low concentrations is more speculative as, to the authors' knowledge, no inhibition of any isolated biochemical system has been described with these low concentrations of phlorrhizin. Lotspeich (1958) has recently discussed a very similar problem in relation to the renal tubular epithelium, i.e. the maintenance of the functional integrity of the cell membrane by the oxidative metabolism of the cell. He outlined various interesting possibilities but was not able to procede further than this. Crane, Field & Cori (1957) have also recently shown that phlorrhizin prevents entry of glucose into ascites tumour cells, but apart from showing that the inhibition is non-competitive no further details of the mechanism were suggested.

SUMMARY

1. A study has been made of the movement of glucose and water by sacs of everted small intestine of the rat.

2. It is shown that phlorrhizin over a wide range of concentrations has an inhibitory effect on movement of glucose and of water.

3. The view is put forward that the main action of phlorrhizin is to prevent movement of glucose into the cells from the mucosal fluid.

4. The behaviour of the intestinal preparation *in vitro* is discussed in the light of this hypothesis, and the mechanisms of absorption of glucose and water by the intestine are discussed.

The authors are indebted to Mr J. Hadfield for construction of the glass apparatus used, and to Mr I. Thorneycroft and Mrs H. Parsons for technical assistance. The expenses of the work were defrayed by grants from the Medical Research Council and from John Wyeth and Brother Limited. Two of the authors (B. J. Parsons and C. B. Taylor) were in receipt of training grants from the Medical Research Council.

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MEASUREMENT OF THE STRIATIONS OF ISOLATED MUSCLE FIBRES WITH THE INTERFERENCE MICROSCOPE

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Observations with the light microscope, both on fresh fibres and on fixed preparations, have led to contradictory views on the changes that take place in the striations of muscle fibres during a contraction. Thus, most of the 19th century microscopists found that moderate degrees of shortening took place by narrowing of the low refractive index bands, or I bands (for references, see A. F. Huxley, 1957, p. 267), while the majority of relevant papers in the present century have claimed that it was the high refractive index (A) bands that shortened during contraction (Engelmann, 1906; Hürthle, 1909; Buchthal, Knappeis & Lindhard, 1936). It is this aspect of the question to which most of our results are relevant, but the experiments were originally planned in the hope of deciding a different point, namely, whether contraction bands are formed, and, if so, under what conditions. The formation of contraction bands, and the consequent reversal of striations, was seen by many observers during the second half of the 19th century (see review by Heidenhain, 1899); this was confirmed by some more recent workers (Jordan, 1933; Speidel, 1939), but denied by others (Hürthle, 1909; Buchthal *et al.* 1936). Even if the reality of these contraction bands was admitted on this evidence, there remained the question whether their formation was an accompaniment of 'activation', of tension development or of shortening; this distinction could not be made on the existing evidence, since only slow contractions, with more or less unlimited shortening, had been studied. We chose fibres from frog muscle as our material, in spite of the fineness of their striation, because it was known that even when isolated they give propagated all-or-none twitches (Ramsey & Street, 1938) in which 'activation' is complete very early after the passage of the action potential (Hill, 1949), while the later development of tension or of shortening could be measured; they therefore afford a possibility of identifying the phase of activity to which any change in the striation pattern is related.

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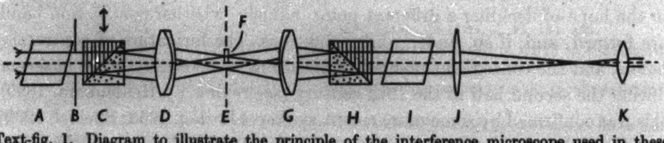
As was known to Abbe (1873, p. 454) and Exner (1887, pp. 383–388) ordinary light microscopy is unreliable on a specimen of this kind. The interpretation of a phase contrast image would also be uncertain, on account of the reversal of contrast which occurs when the instrument is put slightly out of focus; the resultant image would be compounded of, say, a positive image of the structure at the plane of focus, and negative images of planes above and below. Interference and polarization microscopy would both be expected to give more or less satisfactory 'optical sections' of a thick fibre; the former was chosen because the reversal of striations was believed to occur only in respect of the refractive-index difference between the bands, not in respect of their birefringence difference (Engelmann, 1873*b*; Rollett, 1891, p. 90).

The experiments were performed between March 1953 and January 1954, and the main results have already been briefly reported (Huxley & Niedergerke, 1954*a*, *b*).

METHODS

Interference microscope

The microscope was developed by one of the authors with this investigation in view, and has been described elsewhere (Huxley, 1952, 1954). Text-fig. 1 illustrates the principle of the instrument; further components which were necessary to obtain satisfactory performance with a high-aperture objective are described in one of the earlier reports (Huxley, 1954). Two areas in the object plane are illuminated by coherent beams of light obtained by passing the incident light through a polarizer (set at 45° to the plane of the diagram) and a Wollaston double-image prism. The two beams are recombined by a second Wollaston prism above the objective, so that if their optical paths are equal they re-form plane-polarized light which is extinguished by the analyser (crossed with the polarizer). If the light in one of the beams is retarded by passing through a specimen with a refractive index higher than that of the surrounding medium, the recombined light is elliptically polarized with a component which will pass the analyser, so that the specimen is seen bright on a dark background. The amount of the retardation can be measured by compensation exactly as the path difference due to a birefringent specimen is



Text-fig. 1. Diagram to illustrate the principle of the interference microscope used in these experiments. *A*, polarizer; *B*, iris diaphragm; *C*, and *H*, Wollaston prisms; *D*, condenser; *E*, object plane; *F*, specimen, *G*, objective; *I*, analyser; *J*, collimating lens, *K*, eyepiece. From Huxley (1952).

measured in a polarizing microscope, the background path difference for this purpose being introduced by displacing the lower Wollaston prism in the direction of the arrow.

This was done with a micrometer head operated by a lever reading on a scale on which 1 mm was equivalent to 10 mμ path difference; this calibration was checked with the 5461 Å mercury line and found to be correct within 0·5%. Path differences were obtained by setting this lever for minimum brightness at each of the two positions in the field between which a comparison was desired; readings were made to the nearest 0·1 mm (1 mμ path difference) and repeat settings rarely differed by more than 0·2 mm.

The microscope stand was of the type manufactured by Messrs R. and J. Beck for use with their reflecting objectives; its chief advantage for our purpose was that it has a fine focusing movement on the stage as well as on the body, so that the specimen could be focused without changing the separation between objective and condenser, to which the adjustment of the instrument is sensitive. The objective was a Beck 1/7 in. fluorite water immersion, modified by the manufacturers for use without cover-glass; its numerical aperture (n.a.) was 0·88 and its focal length 4·04 mm. Good extinction in an empty field was obtained with illuminating cones up to 0·7 n.a.; cones of 0·5–0·6 were generally used in the experiments described here. The instrument was adjusted to give uniform path difference over the whole field of view (infinite fringe width).

The lateral separation between the two beams in the object plane was 74μ. A fibre narrower than this could be examined in its whole width; with a wider fibre, only a strip of this width along one of its edges could be observed satisfactorily, since the image of the fibre formed by the reference beam obscured the remainder. To reduce scattered light the illuminated field was restricted by an adjustable slit as well as an iris diaphragm. Except when otherwise indicated, the fibre was placed in the beam whose electric vector was parallel to the fibre axis.

The microscope could be converted into an ordinary polarizing microscope by sliding out the lower Wollaston prism. The upper Wollaston prism then acted as analyser, its unwanted beam being absorbed by the analysing polaroid if the latter was rotated through 45°. The lower polaroid acted as polarizer, and compensation could be obtained by inserting a mica λ/4 plate below the condenser and rotating the polarizer. The fibre was turned through 45° by means of the rotating stage but was not otherwise disturbed.

The microscope carried a side tube to which about 15% of the light was diverted by a beam-splitting cube (above the analyser). This side tube held a 20 × visual eyepiece with a micrometer scale. The straight tube carried a 10 × eyepiece accurately placed so as to form a real image on the film in a camera held above the microscope when the specimen was focused through the visual eyepiece.

Photography and illumination

Three cameras could be fixed in position interchangeably on a wall bracket, without mechanical contact with the microscope, as follows:

(1) A still 35 mm camera with focal-plane shutter, in which Kodak 'Panatomic X' film was used; magnification on the film 185 ×.

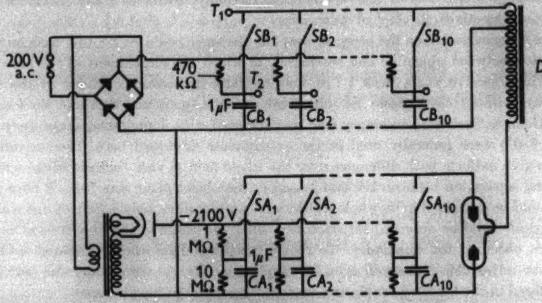
(2) A 16 mm cine camera, with Ilford 'Pan F' negative film, run either at 8 frames/sec with a green filter (Ilford no. 404) or at 16 frames/sec without colour filter; magnification 185 or 263 ×.

(3) For flash photographs, a simple drum camera which rotated continuously carrying a strip 43 × 7 cm of Kodak 'R 55' recording film (orthochromatic), on which ten series each of ten flash pictures 12 mm square (or fifteen series of six pictures) could be taken without reloading. Linear velocity of film about 1 m/sec, parallel to the fibre axis; magnification 182 ×. The green filter was sometimes used.

Visual observations were made with light from a ribbon filament lamp (6 V, 18 A) which was directed towards the microscope by a mirror which could be swung out of the way when photographs were to be taken, allowing light from either a 4·5 A d.c. carbon arc (if cine records were to be taken), or from the flash tube, to reach the microscope. When the arc was used a piece of

Messrs Chance's ON 19 heat-absorbing glass, 3 mm thick, was inserted. A collecting lens was placed in front of each light source, giving Köhler illumination.

The flash tube was the British Thomson-Houston Co's Type FA 5 (xenon-filled). A series of six or ten 1 μF condensers charged to 2000 V could be discharged through it in rapid succession by the circuit shown in Text-fig 2, in which the contacts were closed in turn by pressure from an insulated roller carried on the arm of a Keith Lucas rotary contact-breaker (Cambridge Instrument Co.). The pendulum was released by an electromagnet energized through a contact on the spindle of the drum camera. The fibre was stimulated by pulses from an electronic square-wave generator which could be triggered either by an extra contact which was closed after the first flash or by the discharge of one or more of the condensers *CB* (Text-fig. 2).



Text-fig. 2. Diagram of circuit for flash apparatus. Ten flashes are given by discharge of the condensers CA_1–CA_{10}, each being triggered by the discharge of the corresponding condenser *CB* stepped up by the coil *D*. The contacts *SA* and *SB* are closed in turn by a roller on a swinging arm, so that SA_1 (connecting CA_1 to the flash tube) is closed about 1 msec before SB_1 which applies a triggering pulse from CB_1. SA_1 and SB_1 are opened again before SA_2 and SB_2 are closed in the same way, and so on. Stimulating circuit triggered from T_1 when stimuli synchronized with every flash were required, or from T_2 for stimuli synchronized with a limited number of the flashes; in the latter case the condensers *CB* were charged with the opposite polarity from that shown.

The flash duration was sufficient to cause just appreciable deterioration of the image of an eyepiece cross-line perpendicular to the direction of movement of the film, the extent of the blurring being equivalent to 0·1–0·2 μ at the specimen. The velocity of the image itself, due to movement of the specimen, must have varied greatly from one experiment to another, but in the most successful cases it was less than the velocity of the film, since the same features of the fibre (nuclei, etc.) could be recognized in successive frames. Since the resolving power of an objective of n.a. 0·88 is about 0·4 μ, the effect of flash duration on resolution must have been small, though probably not altogether negligible.

Densitometer

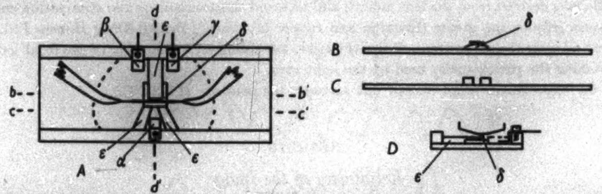
The lengths of the principal bands were measured on the photographic negatives with a micro-densitometer arrangement, using a slit whose width was equivalent to 0·1 μ or less on the fibre. The transmitted light fell on a photocell whose output was amplified and displayed against distance along the film on a cathode ray tube, which was photographed. The lengths of the dark and light bands were measured at a level halfway between the peaks and troughs of the record.

Dissection

Fibres were isolated from the semitendinosus muscles of English frogs (*Rana temporaria*) by a method which did not differ in essentials from those of Asmussen (1932) and of Ramsey & Street (1938). The dissection was carried out at room temperature in a dish made of methacrylate polymer (Perspex), in a Ringer's solution of the following composition (mM): NaCl 111, KCl 2·5, $NaHCO_3$ 2·4, $CaCl_2$ 2·0. Fine scissors were used in the early stages of the dissection, but the separation of the fibres was achieved mainly by cutting the collagen filaments which hold them together with a cataract knife which was pressed down on to the bottom of the dish.

Mounting of the fibre

For experiments on passive stretch and on local contractions, the fibre was held by two pairs of forceps which gripped pieces of tendon left attached to each of its ends. The separation of the forceps could be adjusted by a screw; they could also be moved simultaneously by screw movements along or across the axis of the fibre. The fibre was supported at the point where it was to be observed by lying on a cylindrical glass surface, with radius of curvature 1·0 cm. The arrangement is shown diagrammatically in Text-fig. 3, which also shows the channels through which the fibre was stimulated. For mounting a fibre, the rotating stage of the microscope, with the attached devices for holding the slide and the forceps, was lifted off and placed under a binocular dissecting microscope.



Text-fig. 3. Diagram of arrangements for holding and stimulating the fibre. *A*, plan view of slide with fibre in place, held by forceps. Dotted line is approximate outline of the pool of protein-Ringer in which the fibre lies. *B*, section *b–b'*. *C*, section *c–c'*. *D*, section *d–d'*, with objective in place. In *B–D* the vertical dimensions are exaggerated for the sake of clarity. α, β, γ, Ag–AgCl electrodes for stimulating (β and γ were usually connected together and used as anode); δ, cylindrical lens supporting the fibre opposite the middle of the objective; ϵ, glass pieces stuck on the slide to form a fluid electrode stimulating the fibre at the point of observation.

For isotonic contractions, the tendon at one end of the fibre was hooked on to a spring made of stainless-steel wire, while the other was attached to an isotonic lever made from the hairspring and balance staff of a watch; the tension could be adjusted by moving the regulating lever, to which the hairspring was rigidly fixed. The fibre was observed near the end fixed to the stainless wire spring, at a point where the longitudinal movements during the contraction were small.

For isometric twitches, one end was held with a pair of forceps and the tendon at the other end was hooked on to a needle soldered to the lever of an RCA 5734 electromechanical transducer valve. The needle was coated with shellac, and the case of the valve, to which its anode is connected, was earthed. The valve was operated as a diode (the grid being connected to the cathode) and the load (25 kΩ) was in the cathode circuit. The cathode was condenser coupled to the input of a conventional DC amplifier with a time constant of 1 sec.

Immersion medium

The image of a fibre immersed in Ringer's solution given by the interference microscope is unsatisfactory because (1) the retardation at the centre of the fibre is several wavelengths, so that the image is disturbed by a number of interference fringes parallel to the fibre axis, and (2) rays in different parts of the illuminating cone traverse different thicknesses of fibre substance, and thus are retarded by different amounts so that they cannot all be compensated simultaneously. It was therefore necessary to raise the refractive index of the Ringer's solution to approximate equality with the fibre contents; after unsuccessful attempts with polyvinyl pyrrolidone (in which the fibres did not survive), this was done by adding protein (crystallized bovine plasma albumin, Armour Laboratories Ltd.). In some early experiments the protein was not neutralized and fibres immersed in the acid solution (pH about 5) soon lost their ability to give propagated responses, although they continued for several hours to give strong local contractions in response to depolarization. Unless otherwise stated the experiments described here were performed on fibres immersed in a liquid made by dissolving 0·5 g of the protein powder in 1 ml. 0·1 N-NaOH (which gave a pH of about 7·2) and adding 0·5–0·6 ml. of a solution containing (mM) NaCl 192, KCl 6·65, $NaHCO_3$ 6·35 and $CaCl_2$ 5·3. The amount of the last solution was varied from time to time within the stated range so as to match as closely as possible the mean refractive index of the fibres.

The objective dipped directly into this solution. To prevent the fibres from being poisoned by metallic ions derived from the lens mount, and to avoid short-circuiting the stimulating current, petroleum jelly or tap grease ('paraffin and rubber lubricant', British Drug Houses Ltd.) was smeared all over the metal front of the objective, extending on to the edge of the front lens but not reaching the part actually used by the light rays.

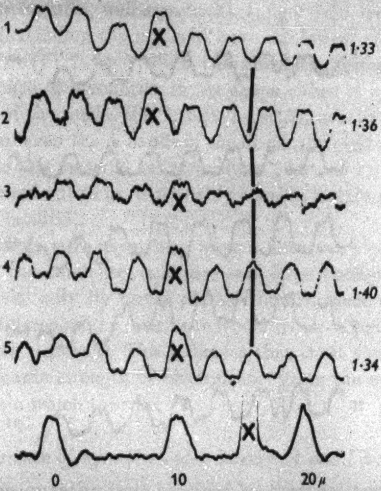
All the experiments were carried out at room temperature (17–21° C.).

RESULTS

Reliability of the image

Effect of altering the compensation. The effect of changing the compensator setting on the image of an isolated frog muscle fibre under the interference microscope is shown by the series of photomicrographs reproduced in Pl. 1, figs. 1–5. The reference beam was retarded by an additional 40 mμ between each member of the series and the next, no other adjustment being made. The top two members of the series are in negative contrast (higher refractive index regions bright) and the bottom two members in positive contrast (higher refractive index regions dark), while fig. 3 is close to the change-over point. The negative images are seen to be closely complementary to the corresponding positive ones, showing that the contrast was genuinely due to interference between the two beams. Densitometer traces taken from the negatives of these photographs are reproduced in Text-fig. 4, and the lengths of the dense (A) bands measured on them are indicated. It will be seen that the A band length derived in this way is the same from a negative as from a positive image, and that it is not sensitive to the exact setting of the compensator in either case.

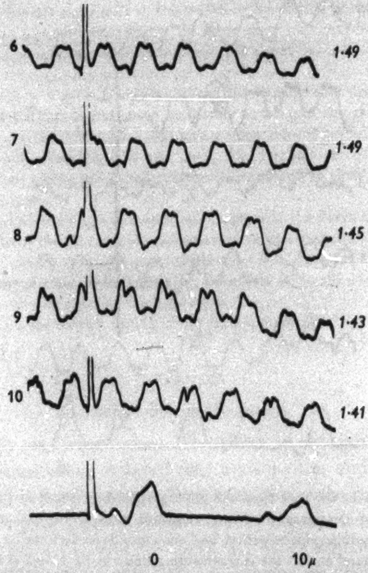
For photographs in negative contrast, a compensator setting intermediate between those corresponding to figs. 1 and 2 of Pl. 1 was used, and for positive contrast, between figs. 4 and 5. Duplicate photographs at positive and negative compensator settings were repeatedly taken during the experiments, and good agreement was regularly found between the A band lengths derived from them. There was, however, a consistent but very slight tendency for the dense band to appear longer in the negative than in the positive image (detectable in Text-figs. 7 and 8), but the difference (about 0·05 μ) was well within the resolving power of the microscope.



Text-fig. 4. Densitometer tracings from the photographs reproduced in Pl. 1, figs. 1–5, showing effect of change of compensator setting. Upward deflexion signals increase of light transmitted through photographic negative, and corresponds to darkness in the print. Each trace starts at the left-hand end of the corresponding print in Pl. 1, and is taken at a level about 1/3 of the way down from the top. Upright lines connect deflexions due to the same A band. Upward deflexions corresponding to the cross-lines are visible; those due to the more nearly vertical line are marked with a cross. Bottom: tracing from photograph of stage micrometer at same magnification. Average length (microns) of A bands indicated on the right for each tracing.

Effect of change of focus. Pl. 1, figs. 6–10, show selected frames from a cine film of a stationary fibre taken as the focusing adjustment was gradually altered without making any other change. The shift between successive members of the series is roughly 10μ. The striations remain very uniform in appearance throughout the series, while nuclei and sarcoplasmic threads come in and out of focus. Densitometer traces from this series are shown in Text-fig. 5, together with the average lengths of the A band measured on them; it is seen that there is no appreciable variation in either amplitude or width of the pattern due to the striations. It was also found that the apparent lengths of the bands was the same at different distances from the edge of a fibre, and was thus independent of the thickness of fibre through which the observation was made.

It may be concluded that the method gave reliable 'optical sections' of these fibres, within the limits set by the resolving power of the system (see p. 418).



Text-fig. 5. Densitometer tracings from photographs reproduced in Pl. 1, figs. 6–10 (numbered to correspond with the plate), showing effect of change of focus. Negative compensation; downward deflexions are A bands. Each trace taken near top of corresponding print in Pl. 1. Bottom: stage micrometer. Average length of A bands indicated on right of each tracing.

Appearance of muscle fibres viewed with the interference microscope

The principal bands (A and I) are thus seen to be distinguished from one another by a considerable difference of refractive index, as has been known since the work of Bowman (1840). In a number of cases, the same fibre was photographed both in interference contrast and by polarization microscopy (for procedure, see Methods section, p. 405) and it was confirmed that the strongly birefringent band (A) was the band with the higher refractive index (Brücke, 1858).

The Z line appeared as a thin line of high refractive index in the middle of I (e.g. Pls. 1 and 2), but was not usually visible at sarcomere lengths below about 2·2μ (see p. 418). The H band was often seen (see p. 413) as a region of lower refractive index in the middle of A (Pl. 2, figs. 2 and 3). The M line, a thin dense line at the centre of A, was occasionally seen in fibres stretched to a sarcomere length of about 3μ. It is just visible in Pl. 2, fig. 4.

These points merely confirm observations made by the classical microscopists, but two other features, well seen in Pl. 1, figs. 6–10, were not expected. First, the nuclei (excluding the nucleoli) have the lowest refractive index of any part of the fibre (Pl. 1, fig. 7; Pl. 2, figs. 1 and 2). Secondly, the material lying between groups of myofibrils, in the shape either of long threads or of caps at the ends of the nuclei (Pl. 1, figs. 7 and 9), usually has a higher refractive index than even the A bands of the fibrils themselves. We have previously referred to this material as 'sarcoplasm', but, as Prof. H. S. Bennett of Seattle has pointed out to us, it probably consists largely of closely packed sarcosomes (mitochondria).

The Z and H bands were never seen when a fibre was viewed by polarized light, so long as the condenser aperture was large enough to ensure that structures were seen only by virtue of their birefringence and not through refractive index differences (i.e. when the whole striation pattern disappeared if the analyser was removed). This indicates that these zones did not differ appreciably as regards strength of birefringence from the other parts of the I or A bands within which they lie.

Difference of refractive index between A and I bands

Estimates of this quantity were obtained by three methods.

On one occasion a group of intrafusal fibres was examined (in Ringer's solution), of which one (thickness 5·5μ, determined by focusing on its top and bottom surfaces) was thin enough for it to be legitimate to assume that the total retardation observed in either A or I band was equal to the product of the fibre thickness and the excess of the refractive index of that band over that of water. The change of compensator setting needed to go from minimum intensity in I to minimum intensity in A was 0·10μ; hence the difference between the refractive indexes of A and I bands was $0{\cdot}10/5{\cdot}5 = 0{\cdot}018$. The sarcomere length was 2·7μ. The mean refractive index of this fibre, estimated in the same way, exceeded that of the surrounding Ringer's solution by 0·06. This is a little higher than that found by more reliable methods on most fibres (see next section), suggesting that the thickness of the fibre may have been underestimated; if so, the value of 0·018 for the A–I difference would also be slightly high.

This procedure could not be employed on the ordinary fibres (thickness 30–80μ) which were generally used for other observations, since it cannot be expected that any large proportion of the rays passing through such a fibre will be confined to a single band during the whole of their passage. The refractive index difference between A and I could, however, be estimated in a different way, in a few fortunate cases where the refractive index of the immersion fluid chanced to be very close to that of either A or I bands. This condition could be recognized by the apparent continuity of the bands of one type with the external fluid when the edge of the fibre was examined.

The difference between the *mean* refractive index of the fibre ($\bar{n}$) and that of the immersion fluid (n_e) was estimated by dividing the fibre thickness into the difference between the compensator settings (*a*) for minimum intensity in the immersion fluid, and (*b*) for equal intensity (near the minimum) of A and I bands at the thickest part of the fibre. Assuming that the mean refractive index $\bar{n}$ is equal to $(l_A n_A + l_I n_I)/s$, where n_A and n_I are the refractive indexes, and l_A and l_I the lengths, of the A and I bands, respectively, and s $(=l_A + l_I)$ is the sarcomere length, it follows that,

when $n_e = n_I$, $\quad n_A - n_I = (\bar{n} - n_e)s/l_A$;
and when $n_e = n_A$, $\quad n_A - n_I = (n_e - \bar{n})s/l_I$.

The results are collected in Table 1. The difference between A and I is seen to average about 0·015, with a suggestion that it increases as the fibre is stretched.

TABLE 1. Estimation of refractive index difference between A and I bands, in cases where one band (indicated in column 2) matches the external solution. A band length assumed, 1·45 μ

Date	Band matched by solution	Refractive index of solution	Sarcomere length (μ)	Maximum path difference (μ)	Thickness (μ)	$n_A - n_I$
9. v. 53	I	1·367	2·6	0·51	55	0·017
15. vi. 53 (*a*)	I	1·371	2·08	0·72	76·5	0·0135
15. vi. 53 (*b*)	I	1·371	3·33	0·52	60	0·0199
15. vi. 53 (*c*)	I	1·371	2·40	0·615	71·8	0·0142
15. vi. 53 (*d*)	I	1·371	3·33	0·445	62·5	0·0164
15. vi. 53 (*e*)	I	1·371	2·5	0·475	70·5	0·0116
1. vii. 53	A	1·377	3·0	0·286	46·5	0·0119
3. vii. 53	A	1·377	3·33	0·625	65	0·0170
		Mean	2·82	—	—	0·0152

A rough estimate of $(n_A - n_I)$ was once obtained in yet another manner. The difference of compensator settings for minimum intensity in A and I was 0·105 μ with the fibre in the beam polarized along the fibre axis (the one normally used) and 0·075 μ with the fibre in the beam polarized at right angles to the fibre axis, both measurements being made at the same point on a fairly thick fibre. These values should be proportional to the values of $(n_A - n_I)$ for the extraordinary and the ordinary ray, respectively. The difference between these values of $(n_A - n_I)$ is the difference between the strengths of birefringence of the A and the I bands, which may be taken to be about 0·004 (taking 0·0022 as a mean of the values in the literature (Høncke, 1947, p. 144) for the mean birefringence of a whole fibre, doubling this and subtracting 10% for the slight birefringence of the I band). Hence, $(n_A - n_I)$ for the extraordinary ray is $0{\cdot}105/(0{\cdot}105 - 0{\cdot}075) \times 0{\cdot}004$ or 0·014, agreeing fairly well with the values found by the other methods.

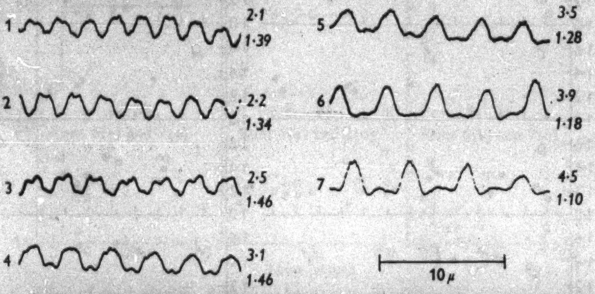
The best value for the difference between the extraordinary refractive indexes of the A and the I bands may thus be taken as 0·015.

Mean refractive index of the fibres

The refractive index of the immersion solutions was determined with a refractometer, and the excess or deficit of the fibre refractive index was found in a number of experiments by dividing the fibre thickness into the path difference. The values obtained on freshly mounted fibres ranged from about 1·36 to 1·39, with a mean of 1·375 for sixteen fibres in the un-neutralized protein solution and 1·378 for eight fibres in the neutralized protein solution. The variation between individual fibres was well outside the errors of the method. The mean refractive index usually fell somewhat during an experiment.

Changes of striations in passive stretch

Lengths of A and I bands. It became clear very early in these experiments that most of the change of sarcomere length took place in the I bands, the length of each A band remaining roughly constant at about 1·4–1·5μ. (The sarcomere length corresponding to the maximum length of the muscle *in situ* is about 2·5μ (Sandow, 1936).) A series of photographs showing this is reproduced in Pl. 2, and densitometer traces made on the corresponding negatives are shown in Text-fig. 6.



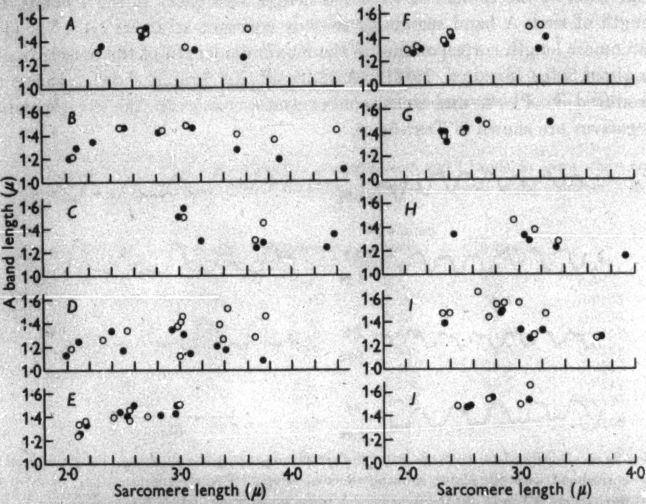
Text-fig. 6. Densitometer tracings from photographs reproduced in Pl. 2, figs. 1–7 (numbered to correspond with the plate), showing effect of stretch. Positive compensation; upward deflexions are A bands. At the right of each trace, upper figure indicates sarcomere length, and lower figure A band length, in microns.

Measurement of such densitometer traces at a level halfway between the peaks and troughs showed only slight variations in A band length, over the whole range of sarcomere lengths from 2·0μ (where the fibres become slack) to 4·5 μ. The results from ten fibres, selected only because they had been photographed at several different degrees of stretch, are plotted in Text-fig. 7. No change in A band length is detectable at sarcomere lengths from about 2·3 to 3·3μ, but both above and below this range there is a consistent tendency for the measured length of A to fall slightly. It is likely that these variations are consequences of the limited resolving power of the optical system; this point will be discussed below (p. 418).

H band. At sarcomere lengths between about 2·1 and 2·9μ, the middle of the A band was seen to be less dense than its edges, constituting an H band

(Engelmann, 1873*a*). This appearance was never seen outside this range. It was not sufficiently sharply outlined to justify measurement.

Appearance of highly stretched fibres. The shape of the densitometer traces of the fibre stretched beyond 3μ per sarcomere in Text-fig. 6 (traces 5–7) is characteristic, with rounded or pointed outlines corresponding to the A bands. It is probable that this is partly due to the M line, which was not seen as a separately resolved structure, except occasionally at a sarcomere length of about 3μ.



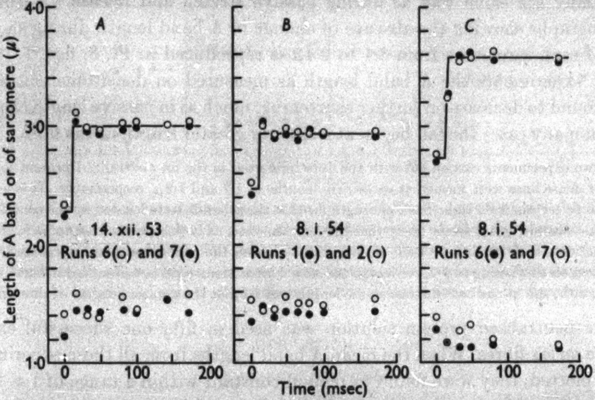
Text-fig. 7. Effect of stretch. A band length (measured on densitometer tracings) plotted against sarcomere length. Each section shows results from one fibre. Open circles, negative compensation; filled circles, positive compensation. *B* is from the same series of photographs as Pl. 2 and Text-fig. 6. *A–G* from stretch series; *H–J* from first frames of several runs with stimulation.

The striation pattern became progressively more irregular as the fibre was stretched beyond a sarcomere length of about 3μ, the bands lying obliquely to the fibre axis and often taking a zig-zag course across the fibre.

Rapid stretch. Several experiments were performed in which the fibre was stretched rapidly during a series of flash photographs. The increase of length was produced by energizing a relay to whose armature one end of the fibre was attached; the movement was timed to begin shortly before the second flash and was complete in about 5 msec. The photographs were traced with the densitometer, and the A band lengths, measured as described above, are plotted in Text-fig. 8. They show no changes greater than were found in slow stretch experiments, even when a flash occurred during the stretch (*C*, Text-fig. 8) or within 3 msec of its completion (*A* and *B*).

Slow contractions

A number of cine films were taken of fibres shortening opposite the cathode of an applied current whose strength was increased gradually over a period of a few seconds (Pl. 3, figs. 1–6). Within the range of lengths where a comparison is possible (sarcomere lengths down to 2μ), the pattern of the striations was found to change with sarcomere length in exactly the same way as has just been described for passive length changes, the length of the A bands staying nearly constant. At sarcomere lengths below 2μ (the minimum reached in passive shortening), two different types of changes were observed on different occasions. Some early experiments (March–April 1953—the protein in the Ringer's solution was not neutralized) showed the sequence illustrated in Pl. 4. On shortening from 2·0 to $1{\cdot}8\mu$ per sarcomere, the dense bands (A) became gradually shorter until they appeared as thin lines; then on shortening to about $1{\cdot}7\mu$ another thin dense line (corresponding to a 'contraction band') appeared midway between each two members of this series. Later experiments, however (January 1954), in which the neutralized solution described on p. 408 was used, did not show these changes, and the ratio between the lengths of the high and low refractive index bands did not change noticeably as the sarcomeres narrowed from 2μ down to $1{\cdot}5\mu$.



Text-fig. 8. Effect of quick stretch. Sarcomere length (upper points) and A band length (lower points) measured on densitometer traces from flash photographs, in three experiments each of which was repeated under identical conditions except that the opposite compensation was used. Open circles, negative compensation; filled circles, positive compensation. Expts. *B* and *C* were on the same fibre.

Isotonic twitches and short tetani

Rapid isotonic contractions were recorded with the flash apparatus and drum camera, the fibre being stimulated by either (*a*) 1 shock applied between the first and second flashes, (*b*) 3 shocks synchronized with the first, third and fifth flashes, or (*c*) 10 shocks, synchronized with each of the 10 flashes. The initial sarcomere length was varied over the range from 2·2 to 3·2μ by altering the tension setting of the isotonic lever, the load during shortening being the same as was used to stretch the fibre before stimulation. It was again found that, whatever the load, the striation pattern varied with length in substantially the same way as during passive stretch and release. A series of micrographs showing the absence of change in A band length during shortening of each sarcomere from 3·1 to 2·4μ is reproduced in Pl. 3, figs. 7–10. In other experiments the A band length as measured on densitometer tracings was found to decrease on further shortening, much as in passive length changes, but in many cases the fall began at a slightly greater length of the fibre.

In two experiments carried out with the fibre immersed in the un-neutralized protein solution, narrow dense lines were formed at sarcomere lengths of 1·7 and 1·9μ, respectively. It is not possible to be certain from these flash photographs that these bands were located at the centre of the original A bands, but it seems likely that this was the case, as it was clearly seen to be in the slow contractions, both by direct visual observation and from the cine films. The intermediate dense lines seen on shortening to 1·7μ or less in the slow contractions were not observed in these experiments, although in one case the sarcomere length was 1·6μ at the greatest degree of shortening.

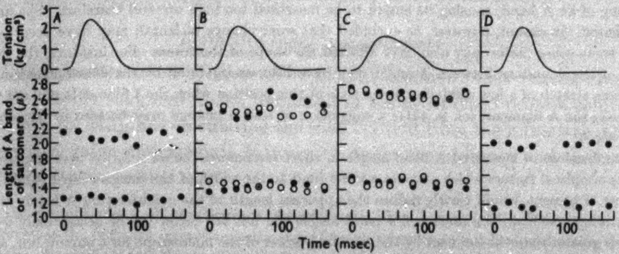
The neutralized protein solution was used in fifty-one successful experiments on six fibres. When the mean A band lengths from all these experiments were plotted, they were found to remain constant within a range of 1·4–1·55μ down to a sarcomere length of 2·4μ, and then fell slowly, though forming an increasing fraction of the sarcomere as far as 1·9μ.

The speed of shortening was about 3–5 muscle lengths per second for fibres near the *in situ* length. This is a little slower than was found by Buchthal & Kaiser (1951, p. 176) on fibres from the same muscle.

Isometric contractions

Series of flash photographs were obtained during isometric twitches, initiated by a stimulus applied synchronously with the first flash. The initial length of the fibre was varied over the range corresponding to 2·1–3·9μ per sarcomere. No consistent change of A band length was found at any stage of the contraction, except that when the fibre was initially fairly short, and the region in the field of view shortened appreciably during the twitch, the A band length fell slightly, with the same relation to sarcomere length as was found in the passive stretch experiments. No other change in the striation pattern was detected. Graphs of A band length, sarcomere length and tension against time, in a representative experiment, are shown in Text-fig. 9.

The peak twitch tensions at moderate extension were in the range from 1 to 2 kg/cm². Tetanus tensions were not measured, but Ramsey & Street (1940) obtained about 3·5 kg/cm² on isolated fibres of the same muscle at room temperature. The observed twitch tensions are therefore high enough to be regarded as normal. The duration of the twitch increased as the fibre was extended, as has long been known for whole muscle (see, for example, Hartree & Hill, 1921).



Text-fig. 9. Isometric contractions. *A–D*, same fibre at different degrees of stretch. Tension records taken immediately after the corresponding series of flash photographs. Single stimulus synchronized with first flash. Upper points: sarcomere length. Lower points: A band length. Open circles, negative compensation; filled circles, positive compensation. Tension in kg/cm², calculated with respect to cross-sectional area at a sarcomere length of 2·5μ.

DISCUSSION

Summary of results

The results which have just been described may be summarized by saying that the width of the A bands stayed nearly constant at 1·4–1·5μ in spite of wide changes of length of the whole muscle fibre, and irrespective of whether the fibre was stimulated or not. This was true whether the length changes were caused by (*a*) slow stretch or release, (*b*) slow local contraction, (*c*) isotonic twitch or tetanus, (*d*) isometric twitch, or (*e*) quick stretch. The formation of contraction bands was observed only in extreme shortening, and then only occasionally. The extent to which these conclusions are supported by other published results, or disagree with them, has been discussed elsewhere (A. F. Huxley, 1957).

The length of the A bands, as measured, did, however, drop somewhat when the sarcomere length was either below 2·3μ or above 3·3μ, and it is therefore necessary to consider what precision can be attached to the measurements before discussing the interpretation of these results.

Accuracy of the measurements

A band lengths. Considering first the range of sarcomere lengths above $3{\cdot}3\mu$, it was observed (p. 413) that the densitometer trace of each A band became dome-shaped when the fibre was stretched to this extent. The measured length of the A bands was therefore more than usually sensitive to the height on the trace at which it was measured; if it were measured at 30% instead of 50% of the height of the A bands in traces 5–7 reproduced in Text-fig. 6, the apparent drop in A band length would disappear. If an M line is genuinely present at these lengths, though not resolved in our micrographs, its effect would be to exaggerate the height of the middle of each tracing of an A band, causing its length to be measured too high up, and therefore to be underestimated. It cannot, however, be excluded that some change of length may have occurred, or that some other factor may also have affected the shape of the traces. For instance, the sharpness of the boundary between A and I may have been exaggerated by the development during extreme stretch of a low refractive index zone at this position when the I filaments pull out from between the A filaments (cf. p. 421); a suggestion of such a change may be seen in trace no. 7, Text-fig. 6.

The decrease of measured A band length in short sarcomeres (below $2{\cdot}3\mu$) is best discussed in terms of optical factors which may set a lower limit to the width of the image of an I band; such factors, if present, would clearly reduce the apparent length of the neighbouring A bands as this limit is approached. The measured I band length did not fall below 0·8 or occasionally $0{\cdot}75\mu$. This is greater than the limit set by the resolving power of the microscope for a narrow line, since, for example, the apparent width of the dense lines sometimes seen in strong contractions (p. 415) was only $0{\cdot}4$–$0{\cdot}5\mu$, which is about the limit due to the numerical aperture of the objective. In the case of an I band, however, a further complication is introduced by the presence of a dense Z line at its centre. This was not visible in these short sarcomeres, as is to be expected since the centres of the two halves of each I band were $0{\cdot}4\mu$ or less apart, and would not have been resolved. The Z line would, however, still flatten the diffraction pattern formed by the I band, and increase its apparent width.

The drop in measured A band width with shortening was somewhat more marked in the flash photographs of isotonic contractions than in the stretch series (p. 416). It is possible that this is due to the resolving power being less good under those conditions. Several factors would tend to produce such a difference: (1) less fine-grained film, (2) movement of film and fibre, (3) the position along the fibre being chosen for minimum movement during the contraction rather than for regularity of the striations, (4) striations sometimes becoming oblique during the contraction.

These appear to be likely explanations for the deviations from constancy of A band length, though it cannot be concluded with certainty that small changes do not occur, particularly in shortened fibres.

Changes in extreme shortening. There seems to us to be no doubt that there was a real difference in behaviour of fibres at sarcomere lengths below $1{\cdot}9\mu$. in the two groups of experiments (p. 415). We have no evidence whether this difference was related to the season at which the experiments were performed, or to the composition of the immersion solution, or to other factors. Nor can we say whether the difference was in the behaviour of the contractile elements themselves, or of other materials in the fibre, though the former seems the more likely. It is possible that this variability has contributed to causing the contradictory opinions that have been expressed concerning the existence of contraction bands, but it is perhaps more probable that this originates from the fact that they are not formed except in extreme degrees of shortening.

The sequence of changes leading to the formation of two sets of narrow lines per sarcomere is closely similar to those described by Jordan (1934) in stained preparations of frog muscle, and by H. E. Huxley & Hanson (1954) in isolated fibrils from rabbit muscle. This phenomenon receives a simple explanation on the 'sliding filament' hypothesis (p. 421), the first set of dense lines being formed by folding of the ends of the secondary filaments when they meet at the centres of the A bands, and the second set (contraction bands) by folding of the primary filaments when they meet at the Z lines.

Refractive index measurements

Mean refractive index of fibres. The mean refractive index (extraordinary ray) for freshly mounted fibres, immersed in the neutralized protein solution, was 1·378 (p. 413). For calculating the solid content from the refractive index, however, a value intermediate between those for the ordinary and the extraordinary rays is needed.

In the case of rodlet form birefringence, the arithmetic mean is appropriate, but with intrinsic birefringence the ordinary refractive index should be given twice as much weight as the extraordinary. Since about two-thirds of the birefringence of muscle appears to be of the former type and one third of the latter (Noll & Weber, 1934), the over-all weight to be given to the ordinary refractive index is $\frac{2}{3} \times \frac{1}{2} + \frac{1}{3} \times \frac{2}{3} = \frac{5}{9}$; the conclusions drawn below are, however, very insensitive to the ratio of form to intrinsic birefringence that is assumed.

Assuming a value of 0·0022 for the total birefringence, the effective mean refractive index is thus found to be $1{\cdot}378 - (0{\cdot}0022 \times \frac{5}{9}) = 1{\cdot}377$. Taking a value of 0·0018 for the specific refractive increment of the solid contents of the fibre (Davies, Wilkins, Chayen & La Cour, 1954; Barer & Joseph, 1954), this is found to correspond to a solid concentration of $(1{\cdot}377-1{\cdot}333)/0{\cdot}0018 = 24{\cdot}4$ g/100 ml.

The water content of whole frog muscle may be taken as 80·3% by weight (see Dubuisson, 1954, Table I). Extracellular water is about 13% of the whole muscle, so that in 100 g of muscle, the cells contain 19·7 g of solids and 67·3 g of water. Taking a value of 0·75 for the partial specific volume of the solids (Cohn & Edsall, 1943), the intracellular volume is $67{\cdot}3 + 0{\cdot}75 \times 19{\cdot}17$, or 82·1 ml., giving a concentration of $19{\cdot}7 \times 100/82{\cdot}1 = 24{\cdot}0$ g/100 ml., with which the figure derived from the refractive index is in excellent agreement.

Difference between A and I bands. The refractive index for the extraordinary ray was found to be about 0·015 higher in the A than in the I band. The corresponding figure for the ordinary ray would be less than this by about 0·004 (see p. 412); giving weights of $\frac{4}{9}$ and $\frac{5}{9}$ to the extraordinary and the ordinary rays respectively (see preceding section), the effective mean refractive index is found to be higher by 0·0128 in the A than in the I band. This corresponds to a concentration difference of $0{\cdot}0128/0{\cdot}0018 = 7{\cdot}1$ g/100 ml.

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The water content of whole frog muscle may be taken as 80·3% by weight (see Dubuisson, 1954, Table I). Extracellular water is about 13% of the whole muscle, so that in 100 g of muscle, the cells contain 19·7 g of solids and 67·3 g of water. Taking a value of 0·75 for the partial specific volume of the solids (Cohn & Edsall, 1943), the intracellular volume is $67{\cdot}3+0{\cdot}75\times 19{\cdot}17$, or 82·1 ml., giving a concentration of $19{\cdot}7\times 100/82{\cdot}1 = 24{\cdot}0$ g/100 ml., with which the figure derived from the refractive index is in excellent agreement.

Difference between A and I bands. The refractive index for the extraordinary ray was found to be about 0·015 higher in the A than in the I band. The corresponding figure for the ordinary ray would be less than this by about 0·004 (see p. 412); giving weights of $\frac{4}{9}$ and $\frac{5}{9}$ to the extraordinary and the ordinary rays respectively (see preceding section), the effective mean refractive index is found to be higher by 0·0128 in the A than in the I band. This corresponds to a concentration difference of $0{\cdot}0128/0{\cdot}0018 = 7{\cdot}1$ g/100 ml.

The corresponding figure that would be expected from the fibrillar proteins alone, if other substances were uniformly distributed along the fibre, may be estimated as follows. About 90% of the dry weight of frog muscle is protein (see Dubuisson, 1954, Table II), and of this 34% may be taken to be 'soluble proteins' (Hanson & H. E. Huxley, 1957). The mean concentration of fibrillar proteins is therefore $0{\cdot}9 \times 0{\cdot}66 \times 24{\cdot}0 = 14{\cdot}3$ g/100 ml. H. E. Huxley & Hanson (1957, Table VI), by interferometric measurements on isolated fibrils, found that the A bands (including H zone) contained 40·5% of the total fibrillar material in excess of what would be required to produce a refractive index equal to that in the I bands. Assuming equal lengths for the A and I bands, the concentration in A would exceed that in I by 81% of the mean concentration, i.e. $0{\cdot}81 \times 14{\cdot}3 = 11{\cdot}6$ g/100 ml. This is substantially higher than the figure of 7·1 g/100 ml. derived above from our refractive index measurements. The difference is too large to be attributed to errors of the method, nor is it lessened by assuming the I bands to be narrower than the A bands; some uncertainty about the validity of this comparison is however introduced by the fact that H. E. Huxley & Hanson's measurements were made on rabbit muscle.

The natural interpretation of the discrepancy, if the comparison holds, is that the I bands contain a higher concentration of soluble proteins and other solutes than the A bands; the presence of a considerable amount of soluble protein in the I bands is suggested by A. F. Huxley & Taylor's (1958) observation of increased contrast when a single I band relaxes after contraction by local activation.

Interpretation of the results

The finding that the A band length was approximately constant under a wide range of conditions immediately suggested that the characteristic features of the band—its high refractive index and its birefringence—are due to the presence there of an array of rodlets whose length does not change when the fibre is stretched or undergoes a contraction. The same conclusion had been drawn long before by Krause (1869) on similar grounds, though this appears to have been generally lost sight of in more recent years; we were unaware of it until a late stage in these experiments.

As to the condition of the I bands, Krause believed them to be fluid, but, assuming on the grounds of electron microscope work (e.g. Hall, Jakus & Schmitt, 1946) that filaments were present, several possibilities were still open. The filaments might be continuous along the whole length of each sarcomere, running between the A band material (Rozsa, Szent-Györgyi & Wyckoff, 1950); they might be highly extensible and attached to the ends of the A band rodlets; they might run between the A band rodlets as far as the beginning of the H band, and there be joined to the rodlets (Hanson & H. E. Huxley, 1953); or they might be more or less inextensible, interdigitating and sliding between the A band rodlets, the gaps between the ends of successive sets of these filaments constituting the H bands, whose width would thus vary with sarcomere length. The last of these possibilities appeared the most attractive to us for several reasons. First, it would explain the fact that the H band was visible only at moderate degrees of stretch; unfortunately we could not measure the width of the H band so as to check this quantitatively. Secondly, the formation of dense lines at the centre of A, on shortening to about 1·8μ sarcomere length, would then be explained as due to folding or overlapping of the ends of these filaments when they came into contact. Thirdly, an explanation would be provided for the linear fall of isometric tetanus tension as fibres are stretched beyond a sarcomere length of a little over 2μ (Ramsey & Street, 1940), if it is further assumed that relative force is generated between the two sets of filaments at sites whose number is proportional to the length of the zone of overlap (Huxley & Niedergerke, 1954*a*).

This scheme was also put forward at the time of our work by H. E. Huxley (1953) on the basis of electron micrographs demonstrating the existence of the two sets of filaments; the crucial demonstration of variation of H band width with sarcomere length was given by H. E. Huxley & Hanson (1954) with phase microscope measurements on isolated myofibrils. The relations between the two sets of filaments has been shown very clearly in more recent work of H. E. Huxley (1957).

Although our results were obtained with an optical system of lower resolving power than has been used on isolated myofibrils, they establish a number of points relevant to this theory of contraction which could only be demonstrated on whole fibres. The following may be mentioned:

(1) The constancy of A band length, and other changes of the striation pattern required by the theory, are demonstrated in fibres still capable of propagated twitches of normal strength, and therefore with the contractile system in a normal condition.

(2) The A band length is shown to be independent not only of fibre length but also of tension and of 'activation' in twitches and tetani.

(3) The A band length is shown to be unaffected by rapid stretch.

The bearing of this theory on various other aspects of muscular contraction has been discussed by one of us elsewhere (A. F. Huxley, 1957).

SUMMARY

1. Changes in the striation pattern of isolated muscle fibres from the frog were investigated by flash- and cine-photography under an interference microscope with numerical aperture 0·88.

2. The length of the A bands was not appreciably altered by passive stretch over the whole possible range (sarcomere length (s) from 2·0 to 4·5μ), the small

drop found at each end of the range being ascribable to errors introduced by the limited resolving power of the optical system.

3. The corresponding increase in I band length took place without delay when the fibre was stretched rapidly (20–30% stretch in 5 msec).

4. An H zone was visible at sarcomere lengths between 2·1 and 2·9μ, but not outside this range.

5. The striation pattern and length of the A bands did not alter at any stage of an isometric twitch, except that, when the part of the fibre in the field of view underwent slight changes of length, the striation pattern altered in the same way as when a similar length change was produced passively.

6. During lightly loaded isotonic tetani, the measured A band length remained constant as the sarcomere length fell from 3·2 to 2·5μ, and then decreased somewhat. This fall may again be due to the limited resolving power, which was somewhat less good in these photographs of moving fibres than in the stretch experiments.

7. In shortening to sarcomere lengths below 1·9μ, two different types of change were observed on different occasions: (*a*), at $s=1{\cdot}8\mu$, dense lines appeared at the centre of each A band, and at $s=1{\cdot}7\mu$, a second set of dense lines (contraction bands) appeared at the positions of the Z lines; (*b*), A and I bands both became progressively shorter, each forming about half of the sarcomere. Contraction bands were only formed under these conditions of extreme shortening.

8. The mean refractive index of the fibres agreed well with the value to be expected from the water content of frog muscle.

9. The refractive index difference between A and I bands corresponded to a difference in protein concentration of about 7 g/100 ml. This is less than would be expected to be produced by the myofibrillar proteins, suggesting that soluble proteins are more concentrated in the I than in the A bands.

10. The observed changes in the striation pattern support the view that length changes, whether produced passively or by contraction, take place at least mainly by sliding movements relative to one another of two sets of filaments, one extending for the length of each A band and the other from each Z line to the beginning of the adjacent H zones.

This work was carried out with the aid of grants from the Rockefeller and Nuffield Foundations. One of us (R.N.) was supported during part of the work by the Deutsche Forschungsgemeinschaft and later by the George Henry Lewes Studentship. We wish to thank Dr R. H. J. Brown for introducing us to the use of flash equipment, and Dr G. S. Adair for help in connexion with the protein solutions.

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EXPLANATION OF PLATES

PLATE 1

Figs. 1–5. Effect of changing compensator setting, from negative in figs. 1 and 2 to positive in figs. 4 and 5; just positive in fig. 3. Setting changed in steps of 40 mμ. Sarcomere length 3·0μ. The fibre has moved about 1·5μ to the left, relative to the cross-lines, during the series, but the individual bands can be identified by reference to the particle adhering to the outside of the fibre at the top left-hand corner of each frame. Densitometer tracings from these photographs are reproduced in Text-fig. 4. Experiment of 23. vi. 53; flash photographs on stationary film (R 55).

Figs. 6–10. Effect of changing focus. Negative compensation (I bands dark). Focus changed by about 10μ between successive pictures of this series, fig. 6 being near the bottom of the fibre and fig. 10 near the top. Densitometer tracings in Text-fig. 5. Experiment of 27. i. 54; selected frames from a cine film. A nucleus is in focus in fig. 7; it appears dark, indicating a relatively low refractive index. The nucleolus, and the cap of interfibrillar material to the right of the nucleus, appear brighter than the A bands, and must therefore be higher in refractive index. Threads of interfibrillar material show up as bright (high refractive index) lines in all the figures; a well-focused example is seen in fig. 9 (arrow).

PLATE 2

Effect of stretch. Positive compensation (A bands dark). The figure beside each frame indicates the sarcomere length in microns. Densitometer traces in Text-fig. 6; A band lengths plotted in Text-fig. 7*B* (filled circles). Experiment of 13. iv. 53; flash photographs on stationary film (Panatomic X). The same nucleus is in the field of view in each frame, but is out of focus in figs. 5–7.

1

2

3

4

5

20μ

6

7

8

9

10

20μ

2·1

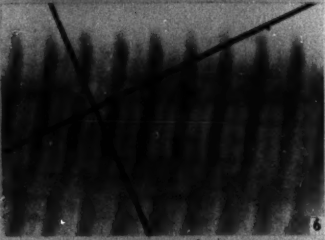
2·2

2·5

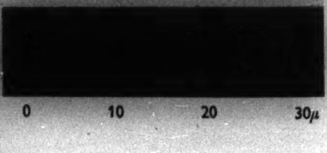
3·1

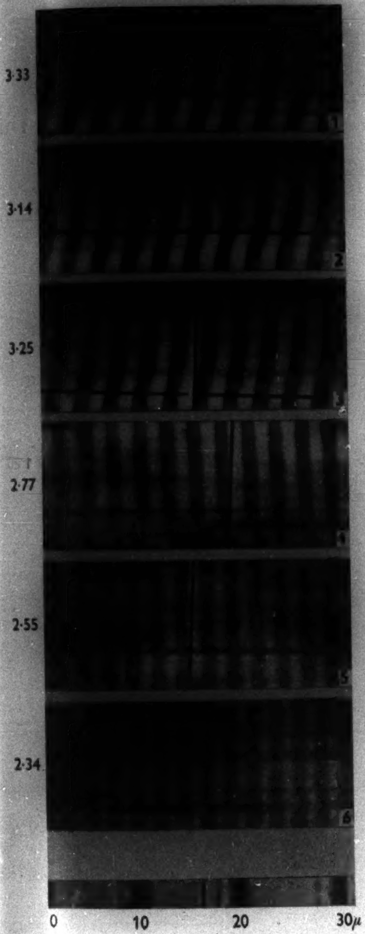
3·5

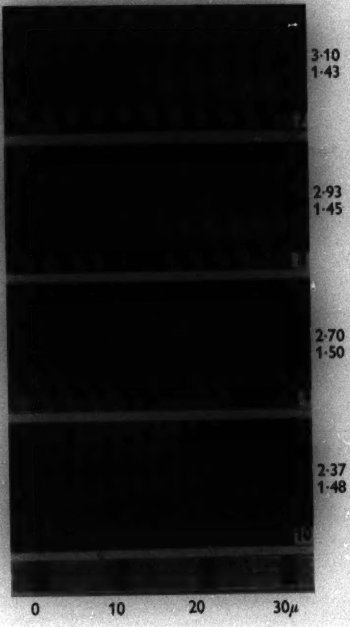
3·9



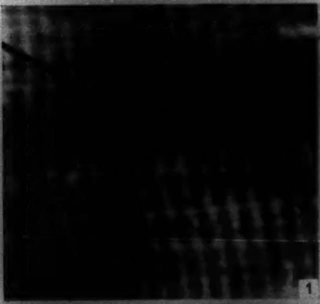
4·5



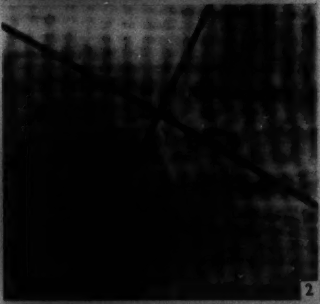

3·33
1
3·14
2
3·25
3
2·77
4
2·55
5
2·34
6
0
10
20
30μ


3·10
1·43
2·93
1·45
2·70
1·50
2·37
1·48
10
0
10
20
30μ

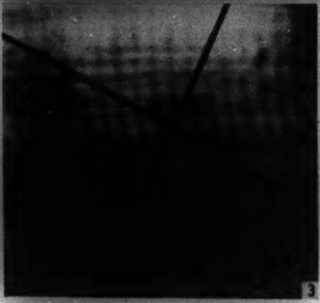
2·20



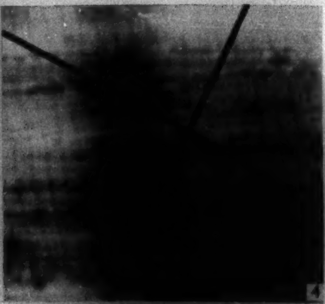
2·04



1·84



1·71



1·60

20μ

PLATE 3

Figs. 1–6. Slow contraction opposite cathode of a slowly rising current. Positive compensation (A bands dark). Sarcomere length in microns indicated on the left of each figure. Experiment of 23. i. 54; from a cine film.

Figs. 7–10. Isotonic tetanus. Frames 1, 4, 7 and 10 from a series of ten flash photographs during an isotonic contraction; stimuli synchronized with each flash. Positive compensation. Figures on the right indicate sarcomere length (upper figure) and A band length (lower figure) in microns, measured on the densitometer traces. Time intervals between the frames reproduced are 52, 48, and 65 msec.

PLATE 4

Slow contraction, showing formation of narrow dense lines first at centre of A bands (figs. 3 and 4) and eventually also at position of Z lines (fig. 5). Positive compensation. Sarcomere length in microns indicated beside each figure. Experiment of 19. iii. 53; un-neutralized protein-Ringer's solution.

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LOCAL ACTIVATION OF STRIATED MUSCLE FIBRES

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The question how the excitation process in a muscle fibre reaches the myofibrils themselves has been discussed from time to time (Engelmann, 1873; Retzius, 1881; Tiegs, 1924), but was raised again in an acute form by Hill (1949). There was already at that time much evidence that the feature of excitation which is causally related to contraction was the reduction (or reversal) of the membrane potential (Kuffler, 1946; Katz, 1950); this in itself could not be expected to affect directly any structures more than a few Angstrom units away from the membrane, so that it was necessary to postulate some link by which myofibrils many microns away from the surface were brought into activity. Current flow in the substance of the muscle fibre appeared to be excluded as the causal agent by the experiments of Kuffler (1946; the conclusion has since been confirmed in other experiments by Sten-Knudsen (1954) and by Watanabe & Ayabe (1956); see also Taylor (1953)). Hill's calculations (1948) showed that the time for diffusion of a hypothetical activating substance, liberated at the surface during the action potential, would be too long to account for the rapidity (Hill, 1949) with which the whole of the contractile material was found to be brought into activity. It therefore seemed that there must be some specific mechanism for this inward spread of activation. The existence of connexions, at the level of the Z line, between adjacent myofibrils, and between superficial myofibrils and the sarcolemma (Enderlein, 1899; Heidenhain, 1911; von Boga, 1937; Draper & Hodge, 1949; Bennett & Porter, 1953), has suggested that this structure may be the route by which the fibrils are activated. The experiments to be described in this paper were begun as an attempt to test this possibility by finding whether contraction could be initiated by depolarizing a small area of membrane lying entirely between the attachments of two Z lines. This was found to be impossible in fibres from the frog (Huxley & Taylor, 1955*a*, *b*), in agreement with the hypothesis, but the interpretation was complicated by the different localization of sensitivity to depolarization that was found in crab muscle (Huxley, 1956). More recent experiments on lizard muscle (Huxley & Straub, 1958) suggest that a component of the sarcoplasmic reticulum (Porter & Palade, 1957) is the structure concerned, rather than the Z line as such.

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METHODS

Principal of the method

The potential difference across the surface membrane of a muscle fibre was altered in a small area, without appreciably changing the membrane potential elsewhere, by applying current to a pipette whose tip, a few microns in diameter, was in contact with the surface of the fibre. The potential of the fluid within the pipette was thus changed, while the current was so small that the potential differences it created in the external fluid outside the pipette, and in the interior of the fibre, were negligible. The pipette was applied to different positions relative to the striation pattern, and the occurrence of contractions was observed microscopically. All experiments were carried out at room temperature (17–20° C).

Localization of potential changes

The order of magnitude of the potential changes elsewhere than within the pipette may be estimated as follows, for the typical case of a pipette of 2μ external diameter in frog Ringer's solution.

Potential differences in external fluid outside the pipette. The distribution of potential around the tip was investigated in a model in which the diameter of the pipette was 3·3 mm external, 2·2 mm internal. The pipette was filled with, and immersed in, frog Ringer's solution, specific resistance $78\,\Omega$.cm. The resistance between the interior of the pipette and a remote electrode was found to increase by $500\,\Omega$ when the tip was brought to a distance of 0·16 mm (about 1/20 external diameter) from a flat insulating surface, representing the surface of the fibre. Assuming that all dimensions change in simple proportion, this corresponds to a resistance of 0·83 MΩ with a pipette of 2μ external diameter (this is about the mean value found) and a conducting layer $0{\cdot}1\,\mu$ thick between pipette and surface membrane. The variation of potential found on the insulating plane in these conditions is shown in Text-fig. 1, falling to a quarter of the maximum at the outside edge of the tip, and to a tenth at a half radius further out.

The maximum value of the potential was roughly equal to

$$i\,(\Delta R + 0{\cdot}38rd),$$

where i = current flowing,

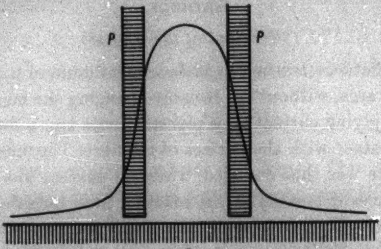
ΔR = increase of resistance of pipette as it is brought into position from a distant point,

r = resistance per unit length of the fluid in the pipette,

and d = external diameter.

Potential changes within the fibre. The area of surface membrane covered by the pipette is about 3×10^{-8} cm^2. Even if it is assumed that the resistance for unit area of membrane, when depolarized by the applied pulse, falls as low as $30\,\Omega$.cm^2, the total resistance of this patch of membrane is $10^9\,\Omega$, and the current flowing for 100 mV depolarization is 10^{-10} A. The total resistance presented by a fibre to current injected at a point is about 1 MΩ (Katz & Thesleff, 1957), so that the electrotonic potential produced within the fibre is of the order of 0·1 mV, which is altogether negligible.

If the area of membrane opposite the pipette undergoes an action potential, it is likely to pass an inward current of about 2 mA/cm^2 (Nastuk & Hodgkin, 1950), making a total current of $0{\cdot}6 \times 10^{-10}$ A, which is again negligible; the threshold strength of current needed to start a propagated action potential is of the order of 10^{-7} A (Fatt & Katz, 1951). The potential change created within the pipette by the action current will also be negligible, since the resistance of the seal formed by the tip of the pipette against the fibre was again of the order of 1 MΩ (p. 434). Similar considerations show that the corresponding disturbances were negligible also in the experiments on crab muscle.



Text-fig. 1. Distribution of potential on the surface of a muscle fibre when a pulse is applied to the pipette, as determined in model experiments (see text). The walls of the pipette, *PP*, and the gap between the tip of the pipette and the surface of the fibre, *F*, are drawn to scale.

Dissection

Frog. Single fibres were isolated from the semitendinosus muscles of frogs (*Rana temporaria*) by a procedure which did not differ in essentials from those of Asmussen (1932) and of Ramsey & Street (1938). Tubocurarine chloride (10^{-5} g/ml.) was added to the Ringer's solution, which was changed a few times during the dissection. Fibres of small or medium size were selected (diameter 40–80 μ approx.). The dissection was carried out at room temperature.

When an excitable fibre was obtained, it was transferred, either in a small scoop containing Ringer's solution or adhering to a thin glass rod, to the trough in which it was to be mounted on the microscope stage. It was held by gripping a piece of tendon at each end with screw-adjustable forceps attached to the mechanical stage.

Crab. Fibres of the extensor of the carpopodite were observed with their attachments intact. A walking leg of a shore crab (*Carcinus maenas*) was detached, and the shell of the meropodite cut away above and below to expose the muscle. The shell of the carpopodite was removed except for a small piece which was tied to the meropodite, so as to hold the muscle fibres at the greatest length they could naturally reach. Fibres were cut away from the muscle until an undamaged and fairly thin one was left at the edge in a satisfactory position for observation under the microscope.

Pipettes

Micropipettes of the type used for intracellular recording (Graham & Gerard, 1946) were drawn on a machine similar to that described by Alexander & Nastuk (1953). These were shortened to provide the required tip diameter by two methods. For the early experiments on frog fibres the tips were broken off in water by rubbing against a fine glass rod with a micromanipulator in the field of a phase-contrast microscope (objective n.a. 1·3). In the later experiments the tips were ground off at an angle of about 60° to the long axis by holding against a carefully cleaned Arkansas stone rotating slowly in a lathe, the pipette being removed frequently for examination under the phase microscope. The pipettes were filled either by boiling under reduced pressure or by means of suction.

Optical apparatus

Polarizing microscope. Many of the observations on frog fibres, and all those on crab muscle, were made with a polarizing microscope. The stand was of Swift's 'Survey' pattern, with Nicol prisms, equipped with a Zeiss D^* water immersion objective (focal length 4 mm, n.a. 0·75) and a Watson 'Holoscopic' condenser with the front lens removed to give a long working distance. The illuminating cone was limited by the polarizing prism to a square whose side (nearly parallel to the fibre axis) gave a n.a. of 0·41. A mica $\lambda/4$ plate was inserted above the polarizer, and the latter was rotated to provide compensation.

Interference microscope. The instrument described by Huxley (1954) was used. This has a water immersion objective of n.a. 0·88, and was used with an illuminating cone of n.a. 0·6–0·7.

With both microscopes the objective dipped directly into the saline surrounding the fibre that was under observation. To avoid short-circuits when current was applied, and to protect the fibre from metallic ions entering the saline from the lens mount, the metal surround of the front lens was coated before each experiment with a thin layer of paraffin and rubber lubricant (British Drug Houses Ltd.). As a further precaution, the objectives were insulated electrically from the microscope body.

Photographic arrangements. The contractions were recorded with a 16 mm cine-camera running at 16 frames/sec. The image was thrown directly on to the film by the microscope eyepiece, giving a magnification of 185 diameters with the polarizing microscope and 320 diameters with the interference microscope, determined by photographing a stage micrometer. A lightly silvered prism below the eyepiece deflected about 15% of the light into a visual eyepiece carrying a micrometer scale, adjusted so that an object focused on the graticule was also in focus on the film. The field was observed continuously during photography, at a magnification of about 800 diameters.

A ribbon filament lamp was used for visual observations, and a carbon arc was switched in for photography by moving a mirror. A heat-absorbing filter was used, but no colour filters.

Ilford 'Pan F' negative film was used.

Micromanipulators

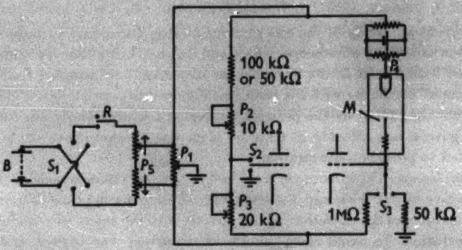
When the polarizing microscope was in use, the pipette was held in a Prior micromanipulator. It was brought into contact with the edge of the fibre, in focus under the microscope, by means of this instrument, and further movements were made by displacing the microscope stand, together with the fibre, by applying light finger pressure to the limb.

When the interference microscope was used, an additional fine movement, parallel to the muscle fibre, was incorporated in the Prior manipulator, and fine vertical movements were made by means of the stage fine adjustment on the microscope, which moved the fibre up and down, leaving the pipette stationary and in focus.

Electrical apparatus

The circuit used is shown in Text-fig. 2. The pulses (about 0·5 sec duration, at intervals of 1·5 sec) were made by closing the relay R, energized by an electronic square-wave generator. With switch S_2 up and S_3 to the left, the circuit became a Wheatstone bridge. This was used to measure the resistance of the pipette by setting P_2 to zero and adjusting P_3 to balance out the deflexion on the oscilloscope screen; the tip of the pipette was then brought into contact with the fibre surface and the increase of resistance determined from the setting of P_2 needed to restore balance. Both grids were kept near earth during the pulse by setting P_1 appropriately. Any d.c. component due to electrode potentials was balanced out by putting S_2 to its down position and adjusting P_4 until the oscilloscope trace was unaffected by changing the position of S_3.

When the effect of local depolarization was to be observed, S_2 was put to its down position and S_3 to the right. In this condition the oscilloscope deflexion was proportional to the current flowing in the pipette.



Text-fig. 2. Circuit diagram. *B*, 18 V dry battery. S_1, reversing switch. *R*, Carpenter high-speed relay. P_5, amplitude control. *M*, pipette. The two valves are the cathode-follower input of a differential direct-coupled amplifier and oscilloscope. For use of the other switches and potentiometers, see text.

Solutions

The composition of the solutions used with frog fibres is given in Table 1.

Solution *A* was used in the earlier, and solution *B* in the later experiments under the polarizing microscope. Solution *C* was used when a sodium-free medium was required.

Solution *D* was used in the experiments under the interference microscope. It contains enough dissolved protein to raise its refractive index to the mean value of the muscle fibres (Huxley & Niedergerke, 1954, 1958). In the experiments on frog fibres, the pipettes were filled with 120 mM-NaCl, except that when solution *C* was in use the pipettes also were filled with this solution. In the experiments on crab muscle the pipettes were filled with 570 mM-NaCl.

TABLE 1. Composition of solutions used in experiments on frog fibres; concentrations in m-mole/kg H_2O unless otherwise stated

Solution	Na^+	K^+	Ca^{2+}	Choline$^+$	Cl^-	HCO_3^-	PO_4 (pH 7)	Dextran* (g/100 ml.)	Protein† (g/100 ml.)
A‡	111·5	2·5	2·0	0	103	15	0	2·0	0
B	117·5	2·5	1·8	0	121	0	1·5	0	0
C	0	2·5	1·8	117·5	121	0	1·5	0	0
D‖	154	2·5	2·0	0	64·2	2·5	0	0	26·6

* 100 ml. of solution A contained 33·3 ml. of 'Intradex' (Glaxo Ltd).

† Crystallized bovine plasma albumin (Armour Laboratories Ltd).

‡ The pH of solution A was brought to 7·2 by adding a small amount of HCl.

‖ The NaOH used in making up this solution (0·2 m-equiv/g protein) brought its pH to 7·4.

RESULTS

Isolated fibres from the frog

Localization of sensitivity to depolarization

The first experiments were made on isolated frog muscle fibres under the polarizing microscope (Huxley & Taylor, 1955*a*, *b*). The fibre was usually stretched to a sarcomere length of about 3μ (some 20% beyond the maximum length in the body). The effect of applying negative electric pulses (duration about 0·5 sec, with intervals of about 1 sec between pulses) to a pipette of 2μ tip diameter in contact with the edge of the fibre is shown in Pl. 1, figs. 1–4.

If the pipette was applied opposite an A band no movement was produced (Pl. 1, figs. 1, 2) unless the strength was raised so high that irreversible changes occurred (see below, p.434). If, however, the pipette was opposite an I band it was often found that each pulse caused a local contraction, as shown in Pl. 1, fig. 4.

It appeared that a contraction was never produced unless the tip of the pipette overlapped the centre of the I band. This is the position of the Z line, but that structure was not visible in frog fibres under the polarizing microscope with the wide illuminating cone that we employed (it can be detected by reducing the cone, but it is then seen in virtue of its high refractive index, not birefringence). In order to check more precisely whether the sensitive point coincided with the Z line, similar experiments were carried out under the interference microscope. It was regularly found that contractions were caused only if the Z line was overlapped by the tip of the pipette.

Localization around the fibre perimeter. This phenomenon of local activation appeared at first to be somewhat capricious: even if the pipette was placed centrally over an I band, it was often found that no contraction occurred, and occasionally an I band became inactive after responding well for some time, or vice versa. The cause of this irregularity was found to be that, as the pipette was moved around the fibre along the line of contact of one Z membrane with the sarcolemma, responses were obtained at some positions but not at others. This was further investigated on the interference microscope, keeping the pipette stationary or moving it only in the horizontal plane while the fibre was moved up or down with the stage focusing movement, which operated with no detectable backlash. Some results of two experiments of this kind are shown in Text-fig. 3. Along any one Z line sensitive regions were found at intervals of about 5μ. These sensitive regions were not at exactly the same level on adjacent Z lines, but in some experiments (e.g. F, Text-fig. 3) an unmistakable correlation was found.

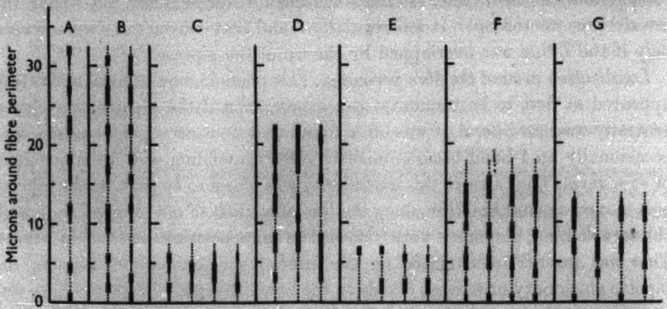
The extent of vertical movement within which responses were obtained consecutively was generally greater than the diameter of the tip of the pipette, suggesting that each sensitive region did not consist simply of a single sensitive spot on the fibre surface. In a few cases the range of movement within which responses were continuously obtained was several times the pipette diameter, showing clearly that the sensitive region was of considerable extent.

Nature, spread and gradation of the response

Nature of the contraction. Whenever a response was seen, it consisted in a movement towards one another of the A bands flanking the I band to which the pipette was applied. The A bands themselves appeared not to change in width during their movement, as is to be expected from earlier observations on frog fibres with the interference microscope (Huxley & Niedergerke, 1954,

1958) and on separated myofibrils under phase contrast (H. E. Huxley & Hanson, 1954; Harman, 1954). The nature of the contraction is well shown in Pl. 1, figs. 5–8.

Symmetry of the contraction. When the interference microscope was used, the Z line was visible, and it was invariably found that this structure remained central in the I band during a local contraction, i.e. the two halves of the I band shortened by equal amounts. This was true even when the edge of the pipette was placed over the Z line, and a weak contraction was produced (Pl. 2, figs. 1–3). In this case, if the two halves of the I band had been activated from different points on the surface, separated by only (say) 0·1μ along the fibre axis, the strength of depolarization they would have experienced would have differed by some 30% (Text-fig. 1), and it would be expected that the amount of shortening in the two halves would have been detectably different.



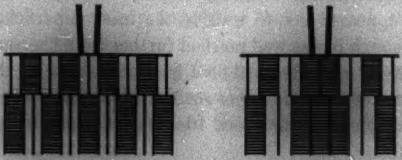
Text-fig. 3. Diagram showing distribution of sensitivity to depolarization at different points along line of contact between Z line and sarcolemma in frog fibres. Each vertical line corresponds to a single Z line; the lines grouped under each letter correspond to adjacent Z lines, tested one after another at each level. Thick full line, well-marked response; narrow full line, weak or doubtful response; dotted, no response. A–D on one fibre; E–G on another.

Asymmetrical contractions were also never seen when a sequence of points along the attachment of one Z membrane to the sarcolemma were tested.

On several occasions contractions were observed at places where a 'vernier formation' was visible a few microns from the edge of the fibre, so that the I band to which the pipette was applied terminated at the vernier opposite an A band. A single position was once found where the contraction of one I band near the edge of the fibre was continued across the vernier, into both the two nearest I bands (Text-fig. 4). Each of these I bands contracted completely, showing that the activation affected both sides of them.

These observations make it highly probable that the half-sarcomeres on either side of each Z line are activated by a single structure.

Movement of water and dissolved material. The shortening of a single I band induced by local depolarization was not accompanied by any detectable expansion perpendicular to the fibre axis. This means that the volume enclosed between the mid planes of the adjacent A bands must have decreased to about half during the contractions, presumably by the expulsion of water. During the first 1 or 2 sec after it had relaxed, the I band was seen under the interference microscope (Pl. 2, figs. 4–6) to have a refractive index markedly lower than its neighbours', indicating that dissolved materials (presumably mostly proteins) had been squeezed out of the I band together with water during the contraction, and returned more slowly than the water after relaxation.



Text-fig. 4. Sketch showing spread of the local contraction across a 'vernier formation' observed on one occasion. Frog fibre; A bands shaded. When the pipette was applied over the adjacent Z lines at the same level, in one case no contraction was obtained, and in the other, a contraction which reached only as far as the vernier formation.

Contraction bands. When a powerful contraction of a single I band was observed under the interference microscope, a dense line was sometimes seen at the position where the two adjacent A bands came into contact. Under the polarizing microscope, however, a thin gap was always seen between the adjacent A bands (Pl. 1, figs. 6–8). These observations are in agreement with the classical descriptions of the formation of contraction bands (Engelmann, 1873; Rollett, 1891) and with recent observations on isolated fibrils (Hanson & H. E. Huxley, 1955, p. 245).

Absence of longitudinal spread. In no case was any shortening observed anywhere except in an I band to which the pipette was applied. This was so even when the contraction spread some 10μ inwards as shown in Pl. 1, figs. 6–8, although the adjacent I bands were only 1–2μ away.

Gradation of inward spread. The speed and extent of the shortening, and usually also the distance to which the contraction spread inwards from the fibre surface, were graded according to the strength of the pulse applied. In our earliest reports (Huxley & Taylor, 1955*a*, *b*) this statement had to be qualified because none of the fibres was capable of giving an all-or-none response to ordinary stimulation at the end of the experiment, and it was possible that a fibre in good condition might have responded to local depolarization with, for example, an all-or-none response involving one I band across the whole cross-section of the fibre. Such possibilities were ruled out in later experiments in which the fibres were still able to conduct action potentials past the point that was under observation, and to give all-or-none twitch responses to ordinary stimulation, after the local contraction had been observed. In all these cases the response to local depolarization was smoothly graded with stimulus strength, and there was no suggestion of all-or-none behaviour.

The greatest distance inwards to which the contraction was observed to spread was about 10μ; it appeared to be easier to obtain a contraction of this extent when using a pipette of 4μ diameter than with one of 2μ. No greater inward spread was obtained with a pipette of 10μ diameter, although several sarcomeres were contracting simultaneously in this case. On a few occasions the contraction appeared not to involve the most superficial layer of myofibrils (1–2μ) although it was well marked further from the surface.

Local contractions were observed on a few occasions with the oblique tip of the pipette in contact with the upper surface of the fibre. The shortening was then also seen to spread up to about 10μ in each direction around the periphery of the fibre.

Response in the absence of sodium

The effect was still present when the sodium of the Ringer's solution was replaced by choline, and the pipette was also filled with this solution.

Magnitude of membrane potential changes

The increase of resistance of the pipette when its tip was brought into contact with the surface of a muscle fibre was measured in most experiments by means of the bridge arrangement described on p. 429. The values obtained are somewhat arbitrary, since they increased as the pipette was pressed harder against the fibre. For a given size of pipette, the values varied over a range of about 2:1 on different fibres, the mean being a little under 1 MΩ for a pipette of 2μ external diameter, and varying inversely with diameter. No change in contact resistance could be detected when the pipette was moved from an I band to a nearby A band, or vice versa.

The potential change at the centre of the pipette was calculated by means of the formula given on p. 429, assuming the internal diameter of the pipette to be two-thirds of the external. Values obtained in this way ranged from 50 to 150 mV when a just-detectable contraction was being elicited, and from 50 to 300 mV when marked contractions, suitable for photographic recording, were produced. These values are likely to be overestimates of the membrane potential change opposite the sensitive spots themselves, since they were obtained, in most cases, without careful adjustment of the pipette in the vertical direction to a point of maximum sensitivity. In addition, there must be a large random error due to variation in the pressure with which the pipette was applied to the fibre.

Effect of overstimulation

The local contractions described so far were reversible, in that the I band extended again as soon as the current through the pipette stopped; they were also repeatable at will at the same point on the fibre. With stronger pulses in either direction, however, irreversible contractions were produced. These differed in the following respects from the reversible contractions:

(1) They persisted after the termination of the applied current.

(2) They involved many sarcomeres.

(3) In many cases when a contraction of this kind was induced in a fresh fibre, the whole fibre moved vigorously, suggesting that the whole or a large part of the fibre underwent a twitch.

(4) If the pipette was kept at exactly the position on the fibre at which it had been when the contraction was initiated, then subsequent application of a positive pulse to the pipette increased the contraction, whereas a negative pulse caused partial relaxation.

These observations are all consistent with the supposition that this type of contraction is produced when the resistance of the surface membrane is drastically reduced as a result of applying too large a potential difference across it, providing a low resistance path from the interior of the pipette to the interior of the fibre. The membrane potential difference (estimated as described in the previous section) that was required to produce a contraction of this kind was a few tenths of a volt; it appeared to be less when the interior of the pipette was made positive than when it was made negative.

Crab muscle

Observations on crab muscle were undertaken in the hope of defining more precisely the positions of the sensitive sites with respect to the striation pattern, since the sarcomere width in the muscle used is 3–4 times as great as in vertebrate muscle. Unexpectedly, the positions were found to be different from those in frog muscle. Observations were made with the polarizing microscope only, and the fibres were immersed in artificial sea water.

Positions of sites sensitive to depolarization. As with frog muscle, a positive pulse produced no result unless it was so strong as to cause irreversible damage, while a negative pulse evoked a strictly localized contraction if the pipette was appropriately placed. In the case of crab muscle, however, the positions of the pipette at which contractions could be initiated were not opposite the Z lines, but at points close to each boundary between A and I bands. There was a suggestion that the sensitive points were just within the A band, and not precisely at the A–I boundary. Pl. 3 shows the pipette at a series of positions where negative pulses were applied, with, in each case, an indication whether a contraction was obtained or not.

Nature of the response. These muscle fibres differed from those of the frog in that the Z line was clearly visible under the polarizing microscope even with the wide illuminating cone that was employed. It was found (Pl. 4) that when a local contraction was produced, the Z line was pulled over to the side of the I band to which the pipette was applied, until it came into contact with the A band. The other half of the same I band never took part in the contraction, and it was usually possible to see that it was actually stretched. No shortening was ever seen in other I bands. The strength of response and the extent of inward spread were graded continuously with the strength of the applied pulse, with no suggestion of all-or-none behaviour.

DISCUSSION

Relationship to normal contraction

There seems no reason to suppose that in these experiments the fibrils are activated by a different route from the one employed in normal contractions. The graded nature of the response is no difficulty, since it has long been recognized (Adrian, 1922; Gelfan, 1933) that the all-or-none character of the twitch of a frog muscle fibre is imposed by the all-or-none electrical response (action potential) of the membrane, and this was eliminated in our experiments as discussed on p. 427. Twitch-like contractions, graded according to the strength of the applied shock, have been recorded by Brown & Sichel (1936) and by ourselves (unpublished) in isolated fibres in which the action potential mechanisms had been lost or eliminated by immersion in a Na-free medium. The normal activity of crab muscle in any case consists largely of continuously graded responses (Katz, 1949). In one of the present series of experiments we applied pulses of a few milliseconds duration to the pipette; the local contraction was obtained and its duration was of the same order as that of a normal twitch.

Limitation of inward spread

Another respect in which these contractions differ from normal twitches or tetani of frog muscle is that they extended only some 10μ inwards from the tip of the pipette, in fibres which might be 70μ in diameter. A simple explanation of this difference is that the influence of local depolarization becomes spread out over a broad front as it progresses inwards and is not maintained in amplitude by any regenerative process. This factor would not arise in a normal contraction when the whole perimeter of the fibre is depolarized in each action potential.

Evidence that depolarization is the effective agent

The strict localization of the effect to the I band (or, in the crab, to the half-I-band) to which the pipette was applied shows that the immediate effect of the applied current on the fibre was at, or very close to, the surface membrane, the inward spread taking place along some specialized structure. This consideration makes it extremely unlikely, for example, that the very small (p. 427) currents in the interior of the fibre could be the causative agent. It seems practically certain that this was the change in potential difference across the membrane itself, as appears to be the case when current is applied to a large area of muscle membrane (Kuffler, 1946; Sten-Knudsen, 1954; Watanabe & Ayabe, 1956).

Relation to Gelfan's observations

Gelfan (1933) recorded graded contractions of single fibres on the surface of a frog's sartorius muscle in response to electric pulses applied to a microcapillary in contact with the fibre surface. These resembled the irreversible contractions that we observed (p. 434) when too large a current was applied, in the following respects:

(1) They involved an appreciable length of the muscle fibre.

(2) Positive current fed to the pipette caused contraction, while negative current reduced the degree of shortening that was already present.

(3) Propagated activity was sometimes initiated at the start of a contraction which subsequently was localized to the neighbourhood of the capillary and was graded with current strength.

It therefore seems fairly certain that the same phenomenon was being observed in both cases. Gelfan explained the reversal of the usual polarity by supposing that the contraction was caused by 'induced cathodes' flanking the anodal capillary; our explanation (p. 435) is equivalent to this, with the added assumption of a drastically reduced membrane resistance opposite the tip of the pipette.

Gelfan did not describe contractions limited to a single striation; it is unlikely that they would have been visible under his conditions.

Mechanism of inward spread

It seems necessary to postulate that the influence of the membrane potential change is conducted inwards along a definite structure related to the striation pattern, in order to explain the localization of the shortening to a single I band (or half-I-band). The discontinuous distribution of sensitivity along the line of contact of a single Z line with the sarcolemma in the frog suggests that the structure in question is a network rather than a complete disk. As to the nature of the influence that is conducted inwards, the only direct evidence provided by our experiments is that the spread is not of an all-or-none kind. It is therefore likely to be a passive process.

The simplest hypothesis consistent with our results is that each fibre contains networks of tubules, electrically continuous with the external fluid, in the transverse planes corresponding to the positions at which surface depolarization is effective (Z line in frog; near to boundaries of A band in crab), and that reduction of the potential difference across the walls of these tubules activates the neighbouring myofibrils. Tubular or vesicular structures forming networks in between the myofibrils have been seen in electron micrographs of muscle from many animals (Bennett & Porter, 1953; Edwards & Ruska, 1955; Robertson, 1956; Porter & Palade, 1957), and in each case the authors referred to have suggested that the structures might be concerned with the conduction of an activating process to the myofibrils. In amphibian (*Amblystoma*) muscle, Porter & Palade (1957) describe at the level of the Z line a characteristic grouping of such tubules or vesicles, which they refer to as a 'triad'. Its position makes it (or one of its components) an attractive candidate for being the inward conductor of the influence of membrane depolarization, but Porter & Palade state that it is not continuous across the fibre, whereas continuity exists between the tubular elements at the middle of the A band.

A further test of the possible role of the triads has been suggested by: (*a*) Porter & Palade's finding that in mammalian muscle there are two triads in each sarcomere, one near each boundary of the A band; and (*b*) Robertson's (1956) description of tubules resembling the centre element of a triad, at similar positions, in muscle from a lizard. If the triads are indeed the structures concerned, it might therefore be expected that lizard muscle would respond to local depolarization more nearly as has been described in this paper for crab muscle than for frog muscle. Huxley & Straub (1958) have carried out the experiment and found that the shortening obtained in this way was confined to one half of the I band when the pipette was placed over the adjacent A–I boundary, the Z line being pulled across into contact with the A band on that side just as we found in crab fibres. Further, one of us (A. F. H.), has confirmed with the electron microscope that structures closely resembling Porter & Palade's triads are present in lizard muscle at levels near each end of the A band. Hence, both in frog and lizard muscle, the region which responds as a unit to local depolarization is centred on the position where triad structures are found, although these positions are very different in the two animals. This suggests strongly that one of the components of the triads is the structure involved, but further study of the tubular elements with the electron microscope, both as regards their comparative anatomy and as regards their continuity and connexions with the surface membrane, is required before this possibility can be regarded as established.

There is little evidence concerning the final step, from these hypothetical transverse networks to the filaments themselves. Some possibilities are discussed elsewhere (Huxley, 1957).

SUMMARY

1. The membrane potential of small areas of the surface of surviving muscle fibres from frog and crab was changed by applying electric current to a pipette whose tip was in contact with the fibre.

2. Reversible contractions, whose strength and degree of spread towards the centre of the fibre varied with the applied current, were produced only if the membrane was depolarized at certain positions in relation to the striation pattern.

3. In frog muscle fibres these sensitive spots were distributed along the line of contact of each Z line with the sarcolemma. The contraction consisted in shortening of both halves of the I band which contained the Z line in question.

4. In crab muscle at maximum *in situ* length, the sensitive spots lay close to each boundary between A and I, probably just within the A band. The contraction consisted in an approximation of the adjacent Z line to the A–I boundary in question, the other half of the I band being unaffected.

5. The contraction never spread longitudinally, although in frog fibres it commonly spread inwards for a distance several times the striation spacing.

6. With stronger pulses of either polarity irreversible local shortening was produced. This agreed in all essential respects with the local contractions studied by Gelfan in 1933.

7. The results on frog muscle, together with those of Huxley & Straub (1958) on lizard muscle, suggest that some component of the 'triads' described by Porter & Palade (1957) in the sarcoplasmic reticulum may be the structure along which the influence of membrane depolarization is conducted inwards.

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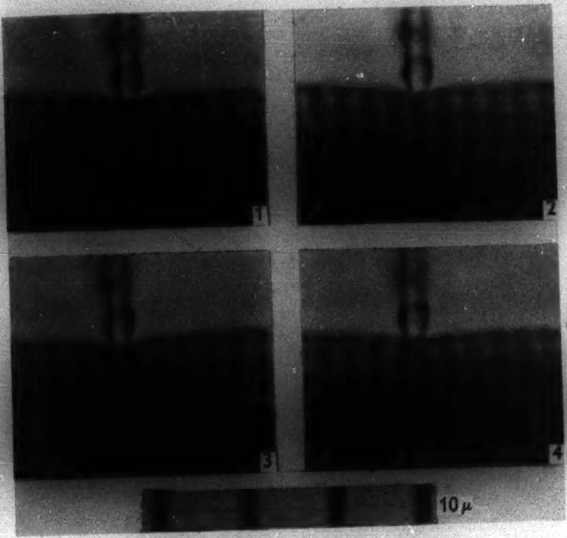
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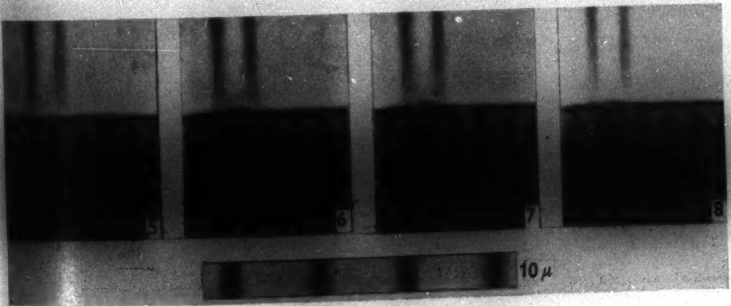
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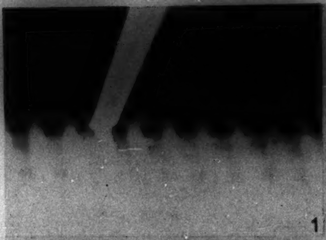

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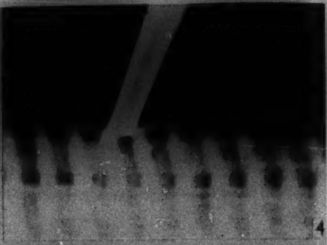

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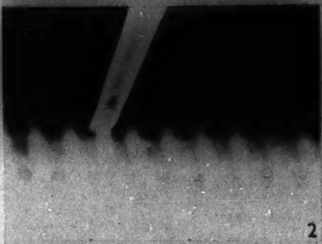

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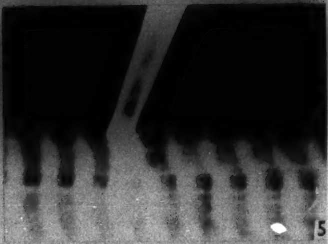

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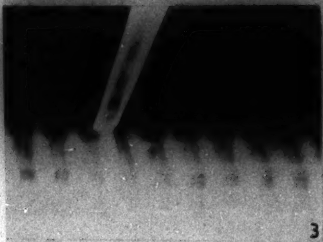

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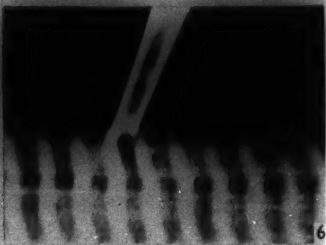

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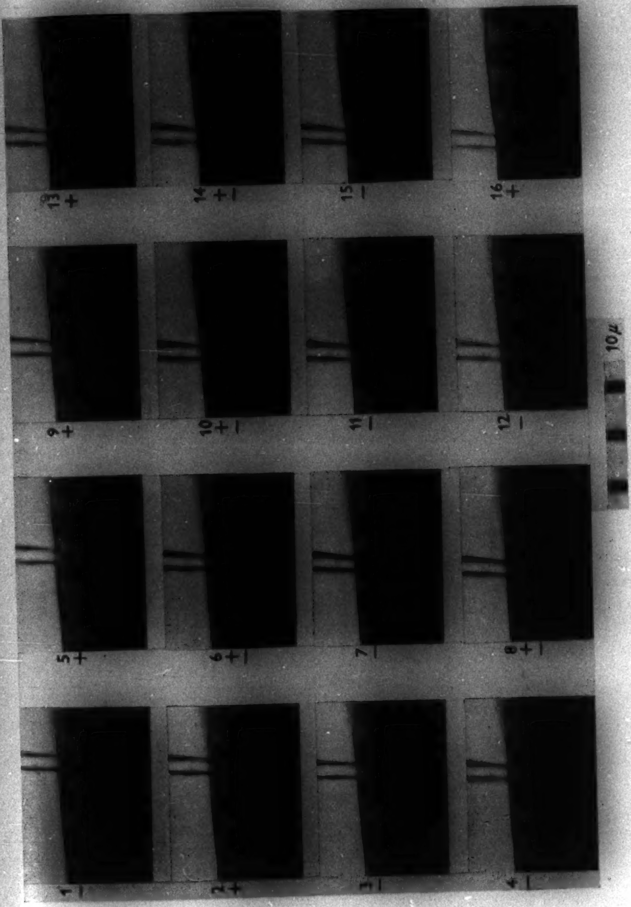
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15 −
16 +
10 μ

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4

EXPLANATION OF PLATES

Plate 1

Figs. 1–4. Edge of isolated muscle fibre from the frog, with pipette in contact. Polarized light, compensated so that A bands appear dark. Pipette applied in figs. 1 and 2 to an A band, and in figs. 3 and 4 to an I band. In each case, the left-hand picture is taken just before, and the right-hand picture during, a negative pulse applied to the pipette; a contraction is produced only if the pipette is opposite an I band (fig. 4).

Figs. 5–8. Successive frames from a cine film (16 frames/sec) showing nature of the shortening induced by local depolarization of a frog fibre; polarized light, compensated so that A bands appear dark. The onset of a negative pulse applied to the pipette occurs between figs. 5 and 6.

Plate 2

Figs. 1–3. Successive frames from a cine film showing that in a frog fibre the response is symmetrical about the Z line. Although the pipette is placed so that its right-hand edge is over the centre of the I band which shortens, the Z line remains central in the I band during the weak contraction which is produced. Interference microscope, compensated so that high refractive-index bands (A and Z) appear bright.

Figs. 4–6. Frog fibre before (fig. 4), during and immediately after (fig. 6) application of a negative pulse to the pipette. Interference microscope, compensated so that high refractive-index bands appear bright. By comparing fig. 6 with fig. 4 it is seen that the I band which has just relaxed is darker (lower refractive index) than before the contraction.

Plate 3

Crab muscle fibre. Polarizing microscope, compensated so that A (and Z) appear dark. Selected frames from a cine film, showing *positions* of the pipette at which contractions were produced (+) or were not (−); ± indicates a weak contraction. Each of these exposures was taken just before the beginning of the test pulse, so that the contractions themselves are not shown.

Plate 4

Nature of the contraction induced in a crab fibre by local depolarization. Polarized light, compensated so that A and Z appear dark. Consecutive frames from a cine film (16 frames/sec). A negative pulse begins between figs. 1 and 2.

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AFTER-POTENTIALS IN MAMMALIAN NON-MYELINATED NERVE FIBRES

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Grundfest & Gasser (1938) described a negative and a positive after-potential following the spike in mammalian sympathetic C fibres. We have studied these after-potentials in an attempt to interpret them in terms of ionic mechanisms.

Frankenhaeuser & Hodgkin (1956) found in giant fibres that the *negative* after-potential is caused by an increase in the potassium concentration around the excitable membrane following activity. The increased potassium concentration occurs because the potassium ions which leak out during activity cannot diffuse freely away from the membrane but are restrained by an external barrier to diffusion. Frankenhaeuser & Hodgkin suggested that this barrier is formed by the Schwann cell layer surrounding the axon. In mammalian non-myelinated fibres a burst of activity also leads to an increased potassium concentration near the excitable membrane (Ritchie & Straub, 1957), suggesting that, as in giant axons, the negative after-potential following a single impulse might be caused by local accumulation of potassium. The vertebrate non-myelinated fibres are contained in invaginations of the Schwann cells (Nageotte, 1922; Gasser, 1955; Hess, 1956; Robertson, 1957*a*). However, calculation shows that the Schwann cell sheath would provide less than 1/200 of the barrier to diffusion required to account for the rate of decline of the negative after-potential, if the gap in the Schwann cell, seen on electron micrographs, were an aqueous phase. This discrepancy may be interpreted as indicating that there is another diffusion barrier in addition to the Schwann cell. This barrier may lie in the space around the axon and the mesaxon, which have been found to contain a highly hydrated gel (Robertson, 1957*b*); moreover, diffusion may be restricted by the amorphous dense material, described as basement membrane (Gasser, 1955; Robertson, 1957*a*), around the Schwann cell.

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Following the negative after-potential there is a *positive* after-potential. The positive after-potential is similar to the larger hyperpolarization which is observed after a burst of repetitive activity. Ritchie & Straub (1957) suggested that the post-tetanic hyperpolarization is caused by an increased active potassium uptake. It will be shown that the positive after-potential after a single impulse can be explained in the same way.

Apart from the negative and the positive after-potentials described by Grundfest & Gasser (1938) we have observed, on preparations of sympathetic C fibres bathed in potassium-free solution and kept at low temperature, a brief period of positivity preceding the negative after-potential. Similar, but longer lasting periods of positivity immediately following the spike have been observed on other types of mammalian non-myelinated fibres and described by Bishop (1934) as the 'positive after-effect associated with the first phase' in vagus nerve fibres, and by Gasser (1950, 1955) as the 'post-spike positivity' in dorsal root C fibres. These periods of positivity observed in various types of C fibres suggest a period of hyperpolarization in the individual fibres identical with the 'positive phase' observed in isolated giant axons (Hodgkin & Huxley, 1939; Curtis & Cole, 1942; Weidmann, 1951). This phase has been explained by Hodgkin & Huxley (1952) on the basis of the persistence of a state of increased potassium permeability after the spike.

METHODS

The technique used was similar to that described by Ritchie & Straub (1956*a*, 1957). Adult lop-eared rabbits were anaesthetized with urethane given as a 25% (w/v) solution into the marginal ear vein (1·6 g/kg). The cervical sympathetic trunk was removed and cleaned under a dissecting microscope. One of the subdivisions of the trunk was isolated and its epineurium removed over a length of about 1 cm, the other subdivisions being cut away. The preparation was mounted in the apparatus described by Stämpfli (1954) and modified by Ritchie & Straub (1956*a*) for recording resting and action potentials from C fibres. The preparation lay in a horizontal insulating capillary 7 mm long, containing sucrose solution, while each end of the nerve was suspended in a vertical tube, through which Locke's solution was flowing. The effects of solutions with different ionic composition (potassium, lithium) were tested by substituting them for the Locke's solution at one side of the sucrose insulator. A pair of Ag–AgCl electrodes, connected over a KCl–agar bridge to the Locke's solution on either side of the insulating horizontal tube, recorded the potential difference between one Locke's–sucrose interphase and the other. The apparatus with the nerve in position is illustrated elsewhere (Straub, 1956). With this technique a high outside resistance is produced between the recording electrodes so that a large fraction of the absolute values of the changes in resting potential and of the slow components of the action potential are recorded. The electrodes were connected to a pair of low grid-current cathode followers and a differential DC amplifier.

The nerve was stimulated through circular electrodes forming part of the inside wall of one of the vertical tubes. The stimulator had an RF output unit (Schmitt, 1948) to isolate the stimulus from earth and to minimize stimulus artifact. The distance between stimulating and recording electrodes was approximately 6 mm.

Monophasic action potentials were recorded from the C fibres after the sucrose solution in the insulating tube had been flowing for about 5 min. The monophasic records were obtained from a defined spot because the conduction of impulses was stopped at the interphase between the

Locke's solution and the sucrose solution, in which lack of sodium ions prevented impulse conduction. This interphase was well defined because small desheathed bundles were used. W[illegible] the usual method of killing one end of an undesheathed nerve by crushing, heating or treati[illegible]th KCl or cocaine it has been found difficult to record monophasically from C fibres (B[illegible]; Gasser, Richards & Grundfest, 1938; Gasser, 1950).

The temperature was measured with a thermocouple bathed in the Locke's s[illegible] millimetres from the Locke's–sucrose interphase on the stimulated side.

The composition of the Locke's solution was: (mM) NaCl, 154; KCl, 5·6; $CaCl_2$, 2·2; $NaHCO_3$, 1·9; dextrose, 5. It was equilibrated with 2% CO_2 and 98% O_2 and had at 30° C a pH of 6·6. The potassium concentration was changed by replacing Na by K; in the Li-Locke's solution LiCl was substituted for the NaCl of the Locke's solution. The sucrose solution contained sucrose (A.R.) 10% (w/v) in distilled water which had been passed over an ion-exchange resin. The sucrose solution had a specific resistance of more than 10^5 Ω cm.

RESULTS

The three types of after-potentials following a single impulse in sympathetic C fibres are seen in Figs. 1–3. Fig. 1 illustrates the negative after-potential which follows the spike in C fibres, when bathed in Locke's solution. On records taken with a slower time base and a higher amplification than that used in the record of Fig. 1, the positive after-potential becomes evident, as is seen in Fig. 2. The brief period of positivity preceding the negative after-potential in preparations bathed in potassium-free solution is illustrated in Fig. 3.

The negative after-potential

Time course. The negative after-potential from C fibres declined approximately exponentially. This is shown in Fig. 4 where the height of a negative after-potential is plotted on a logarithmic scale against time. From about 20 msec up to 150 msec after the spike the points on the graph can be fitted by a straight line; later the slope becomes steeper, presumably owing to the relatively increased contribution of the factors giving rise to the positive after-potential. From ten different nerve bundles thirty-five after-potentials were plotted in this way; they gave results similar to that seen in Fig. 4. The exponential decline of the negative after-potential has also been observed on squid giant fibres by Shanes (1949*a*) and Frankenhaeuser & Hodgkin (1956).

Results of measurements of the time constant of the decline of the negative after-potential in C fibres from the ten nerve bundles are summarized in Table 1. For a given temperature the time constant varied considerably among the ten preparations. The reason may lie in differences in the fibre population amongst the C fibres in the bundle and in the presence of a small proportion of B fibres. These also had a negative after-potential but with time constants shorter than those recorded for C fibres. Since in the conditions of our experiments these B fibres must have been stimulated together with the C fibres, the time constant for C fibres alone would have been somewhat longer than the mean value of 150 msec given in Table 1.

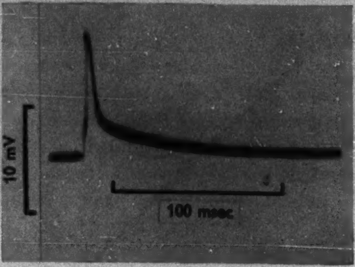

Fig. 1. Monophasic record of the compound action potential set up in a bundle of nerve fibres from the rabbit's cervical sympathetic trunk. The main component of the action potential is a C potential derived from non-myelinated fibres; the other component, the small B potential, is just visible in the rising phase of the spike. The falling phase of the spike is followed by the negative after-potential. Temperature 24° C.

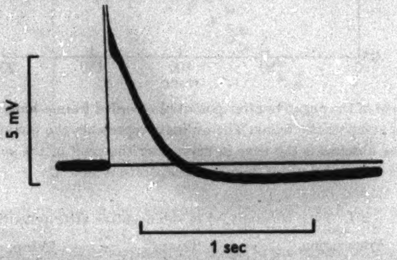

Fig. 2. The negative and the positive after-potentials recorded from a bundle of C fibres from the rabbit's cervical sympathetic trunk. The record was taken with a high amplification so that the spike was too large to be completely registered. The inserted base line shows the position of the resting potential. Temperature 21·7° C.

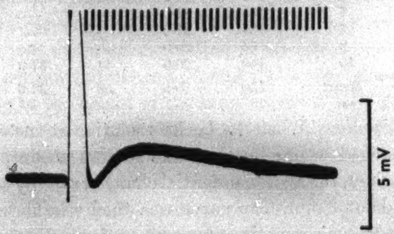

Fig. 3. The 'period of positivity' preceding the negative after-potential, recorded from a bundle of C fibres of the rabbit's cervical sympathetic trunk bathed in a potassium-free solution. Temperature 10·5° C; time 20 msec.

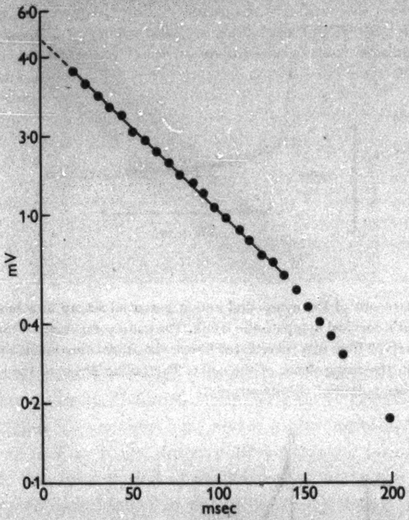

Fig. 4. The time course of the negative after-potential recorded from a bundle of C fibres from the rabbit's cervical sympathetic trunk. The ordinate represents the potential in mV on a logarithmic scale, the abscissa is the time in msec after the peak of the spike. The straight line corresponds to a time constant of 69 msec; temperature 36·5° C.

Table 1. Time constant (τ) of the negative after-potential

Preparation no.	τ (msec)	Temp. (° C)	τ (msec)	Temp. (° C)
1	216	20·0	58	36·8
2	74	21·0	—	—
3	244	20·0	83	37·0
4	138	20·3	50	37·2
5	115	20·8	55	37·0
6	205	21·0	—	—
7	159	21·0	—	—
8	135	21·0	—	—
9	64	20·0	—	—
10	150	20·0	44	37·0
Mean	150	20·5	58	37·0

Effect of potassium ions. When the Locke's solution at one side of the sucrose insulator was changed to one in which the sodium was replaced by various amounts of potassium, the resting potential changed rapidly and reached a new steady level in about 3 min. The nerve was then stimulated and the after-potential recorded; such experiments were performed on five bundles. In Fig. 5 the after-potentials from one nerve bundle bathed in solutions of different potassium concentrations are plotted as in Fig. 4. Changing the potassium concentration had no appreciable effect on the time course of the negative after-potential. The same result was obtained in all five experiments. In each of them the change in resting potential was measured and plotted against potassium concentration. The relation between the potassium concentration and the resting potential was linear from 0 to 12·5 mM potassium. Results similar to these were obtained by Frankenhaeuser & Hodgkin (1956) on giant fibres, suggesting that in both giant fibres and mammalian non-myelinated fibres the same mechanism is responsible for the negative after-potential, i.e. accumulation near the excitable membrane of potassium ions released during the action potential.

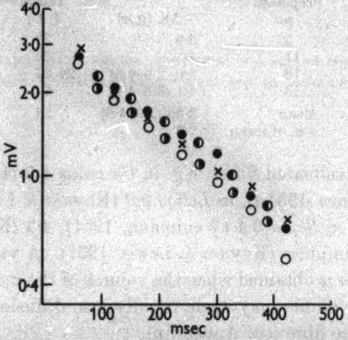

Fig. 5. The effect of potassium ions on the time course of the negative after-potential. The ordinate represents the potential in mV on a logarithmic scale, the abscissa is the time in msec after the peak of the spike. The points represent the height of the negative after-potential recorded when the bundle of C fibres was bathed in solutions with various potassium concentrations (mM): ○, 0·0; ●, 5·6; ◐, 7·5; ◑, 10·0; ×, 12·5. Temperature 25° C.

The increase in potassium concentration near the membrane immediately after the spike can be estimated from the height of the after-potential in Locke's solution, extrapolated to the end of the spike, as shown in Fig. 4, and the curve relating potassium concentration to membrane potential. On this curve a potassium concentration can be found which produces a depolarization equal in magnitude to the height of the negative after-potential at the end of the spike; this concentration gives the potassium concentration at the membrane. By subtracting the initial potassium concentration from this value the increase in potassium concentration is obtained. The results for four nerve bundles are given in Table 2. The mean value for the increase in potassium concentration was 3·35 mM.

Estimation of the net potassium efflux per impulse. The increase in potassium concentration after the spike is likely to be limited primarily to the space between nerve fibre and Schwann cell, which on electron micrographs is about 145 Å thick (Robertson, 1957*a*). Assuming that the potassium ions liberated during the impulse are confined to this space, the net potassium efflux can be estimated by multiplying the increase in potassium concentration (3·35 mM) by the thickness of the space. The potassium efflux estimated by this method would be $4{\cdot}9\times10^{-12}$ mole/cm^2 per impulse at 24° C. This value of 4–5 pmole/cm^2 per impulse for C fibres agrees reasonably well with the potassium efflux measured at approximately the same temperature with other methods on invertebrate non-myelinated fibres, e.g. in *Carcinus* 1·7 (Hodgkin & Huxley, 1947) and 2·4 (Keynes, 1951*a*), in *Loligo* 3·0 (Keynes & Lewis, 1951) and 3·7 (Shanes, 1954) and in *Sepia* 3·4 (Weidmann, 1951), 4·3 (Keynes, 1951*b*) and 3·6 pmole/cm^2 per impulse (Keynes & Lewis, 1951). A value not more than a few per cent higher is obtained when the volume of the mesaxon is also taken into account. This volume may come rapidly into diffusion equilibrium with the space around the fibre (see Appendix).

TABLE 2. Calculated rise in potassium concentration (ΔK) near the membrane at the end of the spike

Preparation no.	ΔK (mM)	Temp. (° C)
3	2·9	22
11	2·8	26
12	3·6 (2·6–4·6)	26–27
13	4·1 (3·8–4·4)	22
Mean	3·35 (2·6–4·6)	24
S.E. of mean	0·3	

Effect of temperature. Lowering the temperature of the Locke's solution prolonged the negative after-potential. This is illustrated in Fig. 6. In three nerve bundles, in which the time constant was measured at different temperatures, an increase of about twofold was found for a 10° decrease in temperature in the range from 38 to 10° C. This is shown in Fig. 7. As these temperature coefficients were obtained from the compound after-potentials the question arose as to whether they were influenced by a change in temporal dispersion resulting from the effect of temperature on conduction velocity. This effect seems to be small in the conditions of our experiments, as shown by the following considerations: if the negative after-potential of all the fibres in the bundle had the same time constant, the time constant of the compound after-potential would be the same as that for the individual fibres and independent of temporal dispersion. We know, however, that this simple case almost certainly does not apply to our results, since the nerve bundles contained a few B fibres, which had a time constant about one third of that of the C fibres. Nevertheless, since the temporal dispersion was small in relation to the observed time constant, it could not have had a large effect on the measured Q_{10}. For a conduction distance of 6 mm which we used the temporal dispersion between

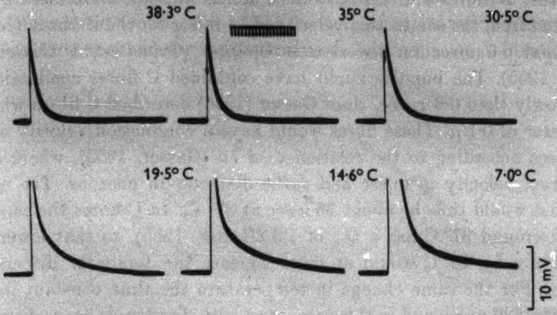

Fig. 6. The effect of temperature on the negative after-potential recorded from a bundle of C fibres from the rabbit's cervical sympathetic trunk kept in Locke's solution. Time 20 msec.

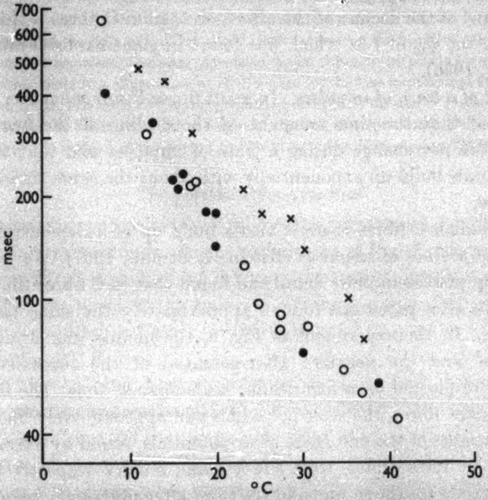

Fig. 7. The relation between the time constant of the negative after-potential and the temperature. The ordinate is the time in msec on a logarithmic scale, the abscissa is the temperature in ° C. The points represent the time constants of the decline of the negative after-potential measured at various temperatures in three bundles of C fibres from the rabbit's cervical sympathetic trunk. Lowering the temperature by 10° C corresponds to a twofold increase in the time constant.

the fastest B fibres and the slowest C fibres was about 10 msec at 37° C, calculated from the conduction velocity of 14 m/sec for the B fibres (Grundfest 1938), and 0·6 m/sec for the slowest C fibres (Grundfest & Gasser, 1938; Gasser, 1955). The bundles might have contained C fibres conducting even more slowly than 0·6 m/sec, since Gasser (1956) described C fibres which had a diameter of 0·1 μ. These fibres would have a conduction velocity of about 0·17 m/sec according to the relation $v=1{\cdot}7d$ (Gasser, 1955), where v is the conduction velocity in m/sec and d the diameter in microns. The maximal dispersion would thus be about 35 msec at 37° C. In C fibres the conduction velocity around 37° C has a Q_{10} of 1·3 (Gasser, 1955), so that lowering the temperature by 10° C would at most increase the temporal dispersion by 11 msec. For the same change in temperature the time constant increased from 60 to 120 msec, and in this case, even with disadvantageous distribution of the time constants, the error in the time constant of the compound after-potential would not exceed 10%. This would cause a 10% error in the Q_{10}, which therefore is between 1·8 and 2·0. The temperature coefficient of the time constant of the decline of the after-potential in C fibres is thus definitely higher than the Q_{10} of 1·34 which was found in giant axons (Frankenhaeuser & Hodgkin, 1956).

The effect of a train of impulses. In giant fibres Frankenhaeuser & Hodgkin (1956) found that the time constant of the decline of the negative after-potential does not change during a train of impulses and that the negative after-potentials build up exponentially with about the same time constant as they decline.

In mammalian C fibres there is also a build up of a depolarization at the beginning of a train of impulses (Ritchie & Straub, 1957). We have studied this build-up process in more detail and found that in C fibres the addition of the negative after-potentials follows approximately the same pattern as in giant axons. In the experiment of Fig. 8, the bundle was stimulated with 6 shocks/sec and the negative after-potential of the successive impulses measured and plotted on a logarithmic scale against time. The figure shows that, as in giant fibres, the after-potentials add approximately linearly. Thus, the initial heights of the first three after-potentials, found by extrapolation as shown by the dotted lines, were 3·0, 4·7 and 5·0 mV respectively, and the calculated values for the second and the third after-potentials found by adding the initial height of the first after-potential to the after-potential remaining at the instant of the second or the third impulse, were 4·8 and 5·6 mV. The difference between the values found by these two methods is small and is probably due to a small error brought in by the extrapolation procedure. Measurements of the slopes in Fig. 8 show that the decline of the negative after-potential becomes faster during a train of impulses. This behaviour of the negative after-potentials in C fibres was also observed by

Grundfest & Gasser (1938). The increase in the rate of decline is probably due to an increased contribution of the factors giving rise to the positive after-potential; they also add during repetitive activity (Ritchie & Straub, 1957).

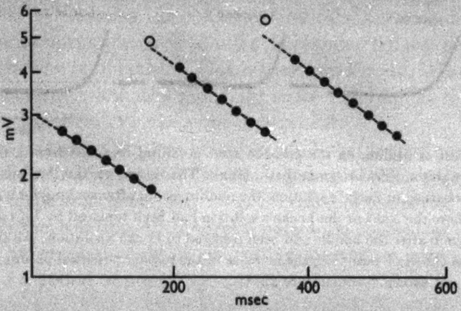

Fig. 8. The addition of the negative after-potentials in the beginning of a train of impulses. The ordinate represents the potential in mV on a logarithmic scale, the abscissa is the time in msec after the peak of the first spike. The closed circles represent the negative after-potentials of three successive impulses. The lines have been drawn through the points by eye. The rate of decline corresponds to a time constant of 316 msec for the first impulse and 290 msec for the second and third. The open circles represent the potential at the end of the second and third spikes obtained by adding the initial height of the first after-potential to the after-potential remaining at the instant of the second and third impulses. Temperature 25·0 °C.

The positive after-potential

The positive after-potential which follows the negative after-potential lasted several hundred milliseconds. In the experiment of Fig. 2, where the records were taken at 22° C, a peak of 0·6 mV over the resting potential was reached 800 msec after the onset of the spike. A similar positive after-potential was recorded by Grundfest & Gasser (1938); it reached its peak at 150 msec and lasted 1–2 sec at 37° C.

Changing the bathing Locke's solution to Li-Locke's solution greatly affected the positive after-potential. The lithium solution increased the resting potential by 3 mV, but did not appreciably affect the spike or the initial height of the negative after-potential. The positive after-potential, however, gradually diminished, as was shown when the nerve was stimulated at 1 min intervals; 5 min after changing to the lithium solution the positive after-potential disappeared. The effect was reversible provided the preparation was not left in the Li-Locke's solution for longer than 15 min. After changing back to normal Locke's solution within this period the positive after-potential reappeared in 2–3 min; it became even slightly larger than before the Li treatment and its peak was reached later. This is illustrated in Fig. 9. When the preparation was left for more than 15 min in the lithium solution a small positive after-potential reappeared whilst the nerve was still in the lithium solution, but there was no full recovery of spike height and after-potentials after changing back to normal Locke's solution.

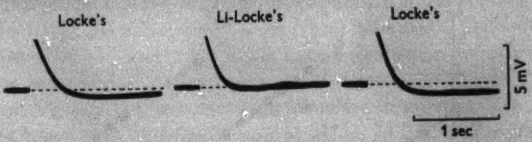

Fig. 9. The effect of lithium on the positive after-potential recorded from a bundle of C fibres from the rabbit's cervical sympathetic trunk. The record on the left was taken when the bundle was bathed in Locke's solution, the middle record after soaking for 9 min in a modified solution where the NaCl of the Locke's solution had been replaced by LiCl and the record on the right 5 min after the bundle had been restored to Locke's solution. All three records were mounted on the same base line; the increase in the resting potential produced by the lithium solution is not shown. Temperature 22° C.

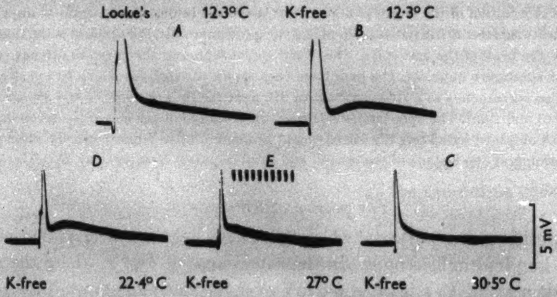

Fig. 10. The effect of potassium ions and temperature on the period of positivity of the negative after-potential. The top record on the left, *A*, was obtained from a bundle of C fibres from the rabbit's cervical sympathetic trunk whilst the bundle was bathed in Locke's solution; the top record on the right side, *B*, which shows a period of positivity, was obtained 3 min after changing to a potassium-free Locke's solution; for both records the temperature was maintained at 12·3° C. The bottom records were all obtained whilst the bundle was bathed in the potassium-free solution. They show that by increasing the temperature the period of positivity disappears. The sequence in which the records were taken was *A* (Locke's at 12·3° C), *B* (K-free solution at 12·3° C), *C* (K-free solution at 30·5° C), *D* (K-free solution at 22·4° C), *E* (K-free solution at 27° C). Time 20 msec.

The initial period of positivity

A period of positivity immediately following the spike in the compound action potential has been described in vagus C fibres (Bishop, 1934) and dorsal root C fibres (Gasser, 1950), but not in sympathetic C fibres (Grundfest & Gasser, 1938). In our experiments, too, no initial positivity was seen when the bundle of sympathetic C fibres was bathed in Locke's solution and kept at 37° C.

However, when the preparation was bathed in a potassium-free solution kept at a temperature below 27° C, there was a period of positivity immediately following the spike. The height of this positivity increased when the temperature was further lowered, and the period of positivity was reversibly abolished either by warming the potassium-free solution to about 30° C or by changing back to normal Locke's solution, containing 5·6 mM potassium, but maintaining the low temperature. This is shown in Fig. 10. When the temperature was sufficiently low, the positivity crossed the initial base line, as is seen in Fig. 3, which is from the same experiment.

Apart from this effect on the after-potential the potassium-free solution caused the well-known increase in the resting potential. This hyperpolarization of the resting membrane is unlikely to be the cause of the period of positivity, since in Li-Locke's solution, with normal potassium concentration, an almost equally high hyperpolarization of the resting membrane occurred but there was no sign of a period of positivity.

DISCUSSION

The negative after-potential

The present results on the negative after-potential in C fibres are consistent with the idea, put forward by Shanes (1949*a*, *b*, 1951) and by Frankenhaeuser & Hodgkin (1956), that the negative after-potential arises from a transient increase in the potassium concentration near the excitable membrane. From the results and the calculations of Shanes, Grundfest & Freygang (1953) and of Frankenhaeuser & Hodgkin (1956) it is clear that the potassium released during a single impulse can account for the height and duration of the negative after-potential in giant fibres only if the diffusion of the potassium ions away from the membrane is restrained by a diffusion barrier in the immediate vicinity of the excitable membrane. In giant fibres Frankenhaeuser & Hodgkin found evidence for a diffusion barrier near the membrane and suggested that it might be formed by the Schwann cell layer which surrounds the axon. For explaining the results of Ritchie & Straub (1957) and the present observations on the negative and the positive after-potential in C fibres some kind of diffusion barrier between excitable membrane and external solution is also required. In C fibres the relation between Schwann cell and nerve fibre is, however, different from that in giant axons: a giant axon is surrounded by a layer of Schwann cells (Geren & Schmitt, 1954), whereas a vertebrate non-myelinated fibre is contained in an invagination of the surface membrane of a Schwann cell, which closely invests the nerve fibre and forms the mesaxon (Nageotte, 1922; Gasser, 1955; Hess, 1956; Robertson, 1957*a*, *b*). Frankenhaeuser & Hodgkin (1956) suggested that in the giant fibres diffusion of potassium ions occurs through cracks in the Schwann cells and thus could account for the rate of decline of the negative after-potential, provided the cracks contain an aqueous phase. If in C fibres the space between nerve fibre and Schwann cell and the mesaxon were to contain an aqueous phase and if we were to assume that the potassium ions diffuse through the mesaxon, the negative after-potential would have to decline with a time constant of the order of 1 msec or less (see Appendix). The observed time constant, however, was about 150 msec at 20° C. This discrepancy suggests that there is another barrier in addition to the Schwann cell. This additional barrier probably lies in the periaxonal space and the mesaxon, which has been found to contain a highly hydrated gel (Robertson, 1957*b*) which may well slow the diffusion of potassium ions; and a further barrier may be formed by the basement membrane which surrounds the Schwann cell.

To explain the effect of temperature on the rate of decline of the negative after-potential in C fibres we have to assume an active uptake of potassium during the negative after-potential. The Q_{10} for diffusion of KCl in aqueous solution is about 1·3, whereas the time constant of the negative after-potential had a Q_{10} of 1·8–2·0. As the potassium ions probably have to diffuse through the gel in the mesaxon and through the basement membrane, the conditions of free diffusion would not be fulfilled and a somewhat higher Q_{10} than 1·3 might be possible, but not as high as 1·8. One may explain the high temperature coefficient by assuming that a relatively large proportion of the released potassium ions returns into the fibres as a result of the relatively low permeability of the outer barrier. This assumption is supported by the fact that an increase in external potassium concentration increases both passive and active potassium uptake in a giant fibre (Hodgkin & Keynes, 1955*a*, *b*). In giant axons the active uptake has a Q_{10} of 3·3, the passive flux a Q_{10} of about 1·0. Thus the high Q_{10} of 2·0 might well result from an active potassium uptake during the negative after-potential. The following calculation supports the idea that the amount of potassium taken up actively during the negative after-potential is considerable and that it could explain the Q_{10} of 2·0. In giant axons the active extrusion of sodium is directly proportional to the external K^+ concentration in the range 0–10·5 mM (P. C. Caldwell & R. D. Keynes, personal communication) as presumably is the coupled active uptake of potassium. If the same were true for C fibres, then the increase in active uptake of potassium, in pmoles/cm², during the interval t_1 after an impulse would be

$$\int_0^{t_1} k_1 y \,\mathrm{d}t = \int_0^{t_1} k_1 y_0 \mathrm{e}^{-t/\tau} \mathrm{d}t = \tau k_1 y_0 [1 - \mathrm{e}^{-t_1/\tau}], \qquad (1)$$

where τ is the time constant of the negative after-potential, k_1 is the constant of proportionality between the rate of active potassium uptake and the external potassium concentration, y, which, immediately after the impulse, is y_0.

The excess potassium in the space, however, is $M = M_0 \mathrm{e}^{-t/\tau}$ pmoles/cm² where M_0 is the value immediately after the impulse. At time t_1, the net quantity $M_0(1-e^{-t_1/\tau})$ has disappeared. Thus the fraction, f, of the excess disappearing by active uptake is

$$f=\frac{\tau k_1 y_0}{M_0}=\frac{100\tau k_1}{\theta}, \tag{2}$$

where θ is the thickness of the space in Ångstrom units.

For giant axons k_1 can be calculated from the data of Hodgkin & Keynes (1955*a*) to be 2·0 pmoles/cm^2.sec per mM external K$^+$ and if it is assumed that the same value is valid for C fibres, equation (2) becomes

$$f=\frac{200\tau}{\theta}. \tag{3}$$

For C fibres $\theta=140$ Å and at τ at 20° is 0·15 sec so that $f=0{\cdot}21$ i.e. 21 % of the disappearance of excess potassium has been by active uptake. With 21 % of the loss having a Q_{10} of 3·3 and 79 % having a Q_{10} of 1·3, a Q_{10} of 1·7 for the negative after-potential would be expected. This is in good agreement with the measured Q_{10} of 1·8–2·0.

In contrast, for giant fibres $\theta=250$ Å and at 20° $\tau=0{\cdot}05$ sec (Frankenhaeuser & Hodgkin, 1956) so that the active uptake would represent only 0·04 % of the total disappearance and thus a Q_{10} of 1·3 would be expected, which again is in agreement with the Q_{10} of 1·3 found for the negative after-potential (Frankenhaeuser & Hodgkin, 1956).

The positive after-potential

The finding that the positive after-potential is abolished when the sodium ions of the bathing solution are replaced by lithium ions enables us to explain the positive after-potential. Lithium ions are able to replace sodium ions in impulse conduction (Hodgkin & Katz, 1949; Gallego & Lorente de Nó, 1951), but on the other hand lithium ions which enter cells passively are pumped out at a much slower rate than are sodium ions (Harris & Maizels, 1951; Swan & Keynes, 1956). Thus the observation that the positive after-potential disappears in lithium solution and reappears upon addition of sodium ions links the positive after-potential with the active extrusion of the sodium ions which have entered the axon during the impulse.

Previous work on the hyperpolarization following a burst of activity showed that similarly the post-tetanic hyperpolarization is abolished in lithium solution and re-appears upon addition of sodium ions (Ritchie & Straub, 1957). The positive after-potential and the post-tetanic hyperpolarization can therefore be considered to be caused by the same mechanism. Ritchie & Straub (1957) calculated that a burst of activity is followed by a *large* increase of the sodium concentration inside a small nerve fibre, which could explain the post-tetanic hyperpolarization. For the positive after-potential following a single impulse the following considerations apply: in giant fibres which have a diameter of 30–500 μ, the sodium influx of 4 pmoles/cm^2 during one impulse (Hodgkin, 1951) increases the internal sodium concentration by only 0·005–0·0003 mM or by 0·01–0·0006 % of the normal internal sodium concentration; accordingly the larger nerve fibres of *Sepia* or crustacea have no positive after-potential, unless tetanized at a high rate (Bugnard & Hill, 1935; Shanes, 1949*b*). For C fibres the sodium influx per impulse has not been measured, but we can assume that it is near 5 pmole/cm^2, since we found that the potassium efflux in these fibres which is approximately equal to the sodium influx was about 4·9 pmole/cm^2 per impulse. Thus the ion fluxes are approximately the same in large and in small non-myelinated fibres; on the other hand, the change in internal sodium concentration is inversely proportional to the fibre diameter. A single impulse would therefore increase the sodium concentration by 2 mM in the smallest fibres of 0·1 μ diameter (Gasser, 1955, 1956), and by 0·2 mM in the larger fibres which have a diameter of 1 μ. The normal sodium concentration in mammalian C fibres is not known, but in mammalian myelinated fibres, Krnjević (1955) found a sodium concentration of 40 mM. This value might be too high, but even with this value one impulse would still increase the sodium concentration by 5 % in the smallest and by 0·5 % in the largest C fibres. Further, Hodgkin & Keynes (1955*a*, 1956) found that the rate of sodium extrusion is proportional to the inside sodium concentration and that about 2/3 of the sodium extrusion is coupled with potassium uptake. In C fibres a single impulse would therefore be sufficient to cause a significant increase in the rate of sodium extrusion and potassium uptake. The positive after-potential can therefore be explained by the same mechanism which is responsible for the post-tetanic hyperpolarization (Ritchie & Straub, 1957), i.e. that the faster potassium absorption, coupled with the increased rate of sodium extrusion, decreases the potassium concentration in the vicinity of the membrane below the concentration maintained in the steady state before the impulse and thereby increases the membrane potential.

The initial period of positivity

A positivity following the spike, similar to that which we have found to occur in sympathetic C fibres, has been described in other types of non-myelinated fibres, e.g. in mammalian vagus fibres (Bishop, 1934) in mammalian dorsal root C fibres (Gasser, 1950, 1955) and in single, isolated cephalopod giant fibres (Hodgkin & Huxley, 1939; Curtis & Cole, 1942; Weidmann, 1951). The period of positivity observed in the three types of mammalian non-myelinated fibres and the positive phase in giant fibres can be considered to be the same phenomenon. This conclusion is based on the following observations, in which a similar behaviour of the period of positivity in the three types of mammalian fibres and of the positive phase was found: (1) During the period of positivity and during the positive phase the fibres are in a refractory state. This was shown for dorsal root fibres by Gasser (1950) and for giant fibres by Hodgkin & Huxley (1952). (2) Both the period of positivity and the positive phase are dependent on the potassium concentration in the bathing fluid. In sympathetic C fibres we obtained a period of positivity only in potassium-free solution and the positivity disappeared when potassium was added to the bathing fluid; in giant axons the positive phase was found to be greatest in potassium-free solution and to disappear when the solution contained a high potassium concentration (Hodgkin & Katz, 1949; Hodgkin & Keynes, 1955*b*). (3) A train of impulses reduces the positivity: this was found in dorsal root C fibres by Gasser (1950) and in giant fibres by Frankenhaeuser & Hodgkin (1956). And (4) a rise in temperature reduces the positivity in the compound after-potential of a population of fibres. This was found by Gasser (1950) in dorsal root C fibres and in the present experiments on sympathetic C fibres.

In giant fibres the positive phase has been explained in terms of increased potassium permeability (Hodgkin & Huxley, 1952). There is evidence in these fibres that the potassium permeability increases during the second half of the spike and does not at once return to its resting value. The resting potential of an isolated axon is several millivolts below the equilibrium potential for potassium ions and therefore the persistence of the increased potassium permeability during the refractory period raises the membrane potential and generates the positive phase. In potassium-free solution the difference between the equilibrium potential for potassium and the resting potential is greatest and this explains why the positive phase is most pronounced in this condition. The period of positivity in mammalian C fibres is explained on similar lines, because it is also associated with a refractory period and because the relationship between resting potential and external potassium concentration (Ritchie & Straub, 1957) is similar to that in cephalopod axons (Curtis & Cole, 1942; Hodgkin & Keynes, 1955*b*).

The difference between sympathetic and dorsal root C fibres. We have concluded that there is no basic difference between the positive phase in an individual sympathetic C fibre and the positive phase in an individual dorsal root C fibre. It is clear, however, that the compound after-potentials recorded from a population of sympathetic C fibres or of dorsal root C fibres are different. Thus, at 37° C, in sympathetic C fibres the compound after-potential shows no period of positivity, even when the preparation is bathed in potassium-free solution, whereas, at the same temperature, in dorsal root C fibres the spike is followed by a large long-lasting positivity (Gasser, 1950, 1955, 1956). It has been suggested that the difference between these after-potentials is so important that it necessitates the division of the C fibres into two sub-groups, the sympathetic C fibres (s.C) and the dorsal root C fibres (d.r.C) (Gasser, 1950, 1955). The relation between nerve fibre and Schwann cell cannot explain this difference, since the relation is the same (Gasser, 1955) in both types of fibres.

On the assumption that the positivity in an individual C fibre is caused by the same mechanism as the positive phase in a giant fibre the difference between these compound after-potentials can be explained by the difference in the duration of the refractory period. It is short, about 20 msec, in sympathetic C fibres, and unusually long, about 300 msec, in dorsal root fibres at 37° C (Grundfest & Gasser, 1938). Accordingly the positive phase in an individual sympathetic C fibre is brief, whereas in a dorsal root fibre it is long. Considering the temporal dispersion between the positive phase of the individual fibres this difference could produce the observed difference in the compound after-potentials. The long-lasting positivity in dorsal root fibres sums, as shown by Gasser (1950, 1955), and will therefore be revealed as a period of positivity in the compound after-potential. On the other hand, in sympathetic C fibres the brief positive phase of the individual fibres is masked by the long-lasting negative after-potential so that no positivity is seen in the compound after-potential. The observations on the effect of temperature on the period of positivity in the compound after-potential support this suggestion. Lowering the temperature decreases the rate of the permeability changes for potassium and sodium with a temperature coefficient of about 3·0 (Hodgkin, Huxley & Katz, 1952); lowering of temperature therefore greatly increases the duration of the positive phase and consequently tends to synchronize the positive phase of the individual fibres. The effect is seen in our experiments on sympathetic C fibres as well as in Gasser's experiments on dorsal root fibres by the increase in the height of the positivity which occurs on lowering the temperature. It might be argued that decreasing the temperature has the additional effect of decreasing conduction velocity and thereby increasing temporal dispersion. This effect, however, is small, as judged from the low Q_{10} of 1·3 for conduction velocity around 37° C (Gasser, 1950); furthermore, in C fibres lowering the temperature has a greater effect on the duration of the action potential than on the decrease in conduction velocity (Ritchie & Straub, 1956*b*).

Thus there seems to be no need for postulating a basic division between sympathetic and dorsal root C fibres, but the difference in the refractory periods ought to be kept in mind when other differences in the behaviour of these two types of C fibres are considered.

SUMMARY

1. Monophasic action potentials were recorded from desheathed bundles of mammalian sympathetic C fibres, kept in flowing saline solutions, with the sucrose-gap method.

2. The spike of the compound action potential was followed by a negative and a positive after-potential.

3. The negative after-potential declined approximately exponentially with a time constant of 150 msec at 20° C; the rate of decline was independent of the potassium concentration in the bathing fluid. The time constant of the decline had a Q_{10} of about 2·0.

4. In the beginning of a train of impulses there was an exponential build up of a depolarization which had approximately the same time constant as the decline of the negative after-potential.

5. It is suggested that in C fibres the negative after-potential is caused by the same mechanism as in giant fibres, i.e. by a transient increase in the potassium concentration in the vicinity of the excitable membrane.

6. An increase of about 3·3 mM in the potassium concentration near the membrane would account for the initial height of the negative after-potential. On the assumption of a uniform distribution of this increase in potassium concentration in the space between nerve fibre and Schwann cell, the net potassium efflux was estimated to be $4{\cdot}9 \times 10^{-12}$ moles/cm² per impulse.

7. The positive after-potential had a duration of several hundred milliseconds. It was reversibly abolished when the nerve fibres were bathed in a solution in which the sodium chloride had been replaced by lithium chloride.

8. It is suggested that the positive after-potential results from an increase in the rate of active potassium uptake following an impulse.

9. In potassium-free solution at a temperature below 27° C the spike was followed by a brief period of positivity preceding the negative after-potential. This brief period of positivity results from a period of positivity in the individual fibres which is suggested to be identical with the positive phase in giant axons.

10. The difference seen in the compound after-potential recorded from a bundle of sympathetic or dorsal root C fibres is explained by the difference in the duration of the refractory periods in the two types of C fibres.

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APPENDIX

Calculation of the time constant expected on the basis of free diffusion of K⁺ ions away from a C fibre

Fig. 11 shows a schematic diagram of the axon (cf. Robertson, 1957*a*). B represents the boundary between the peri-axonal space and the mesaxon and B' that between the mesaxon and the bulk of the solution. For the calculation we assume a fibre of 1·5 μ circumference ($2L$), corresponding to a diameter of about 0·5 μ. For simplicity in solving the diffusion equation we assume the thickness, θ, of the space between axon and Schwann cell to be one half the thickness, 2θ, of the mesaxon; this assumption should not affect the order of magnitude of the results. It is further assumed that the negative after-potential is proportional to the average excess concentration of potassium in the peri-axonal space bounded by the surface B. Since the half circumference $L\,(=0{\cdot}75\ \mu)$ is much greater than $\theta\,(\simeq 0{\cdot}014\ \mu)$, Fig. 11 is equivalent to the diffusion system shown in Fig. 12, where the sides and left-hand boundary are impermeable surfaces and where the peri-axonal space is represented by the length L and the mesaxon by the length N.

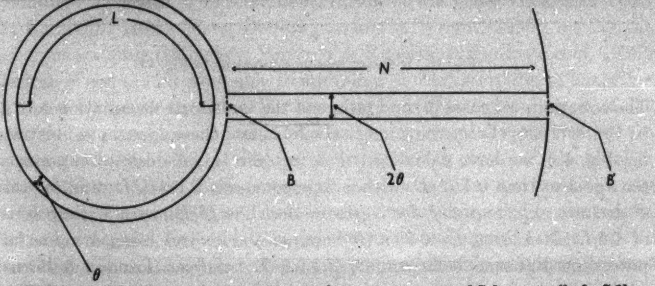

Fig. 11. Schematic diagram of the axon, peri-axonal space, mesaxon and Schwann cell of a C fibre. For clarity, the thicknesses of the peri-axonal space and mesaxon are drawn on a larger scale than is the axon.

It is assumed that immediately after the impulse the excess concentration of K^+ is uniform in the peri-axonal space and zero in the mesaxon, and that at all times the space to the right of B', representing the bulk of the solution, is maintained at zero excess. From the solution to the diffusion equations with the boundary conditions stated above, the K^+ flux across the surface B can be calculated as a function of time and the total loss of K^+ is then given by integration with respect to time. These calculations were made for the three cases of mesaxons of length 0, L and infinity. The fraction, F, of the initial excess remaining at time t is then given by:

(*a*) For $N=0$

$$F=\sum_{n=0}^{\infty}\frac{8}{(2n+1)^2\pi^2}\exp\left(-[2n+1]^2\pi^2Dt/4L^2\right),$$

(*b*) For $N=L$

$$F=\sum_{n=0}^{\infty}\frac{8}{(2n+1)^2\pi^2}\exp\left(-[2n+1]^2\pi^2Dt/16L^2\right),$$

(*c*) For $N=\infty$

$$F=1-\sqrt{(Dt/L^2)}\,\{1/\sqrt{\pi}-ierfc\sqrt{(L^2/Dt)}\},$$

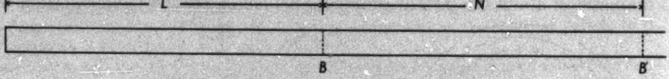

Fig. 12. Diffusion system equivalent to Fig. 11.

The equations for cases (*a*) and (*b*) are of the same form as equation 4.18 given by Crank (1956). Using the numerical solutions to these equations, illustrated in his Fig. 4.6, we have calculated that for case (*a*), F declines exponentially, for t greater than 0·1 L^2/D, with a time constant of 0·4 L^2D; and for case (*b*) F declines exponentially, for t greater than 0·4 L^2/D, with a time constant of 1·6 L^2/D. Taking $L=0{\cdot}75\times10^{-4}$ cm, as above, and using a value for the potassium diffusion coefficient, D, of $1{\cdot}5\times10^{-5}$ cm²/sec (Landolt & Börnstein, 1935), the excess potassium in the peri-axonal space disappears with time constants of 0·15 and 0·60 msec respectively, whereas the experimental value was 150 msec. In case (*c*), the rate of disappearance of excess potassium never becomes exponential. Moreover, the rate is too rapid until almost all the excess potassium has disappeared. Thus, using the values of L and D given above, F decreases from 1·0 to 1·0/e in 0·75 msec and from 1/e to $1/e^2$ in 5·4 msec.

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CONTINUOUS AND REFLEX SECRETION BY THE PAROTID GLAND IN RUMINANTS

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The parotid glands of adult ruminants secrete saliva rapidly and continuously. The large volume of alkaline saliva produced helps to maintain the relatively constant pH and the fluid nature of the fermenting contents of the rumen. The rate of secretion varies; it increases particularly during feeding and rumination (Colin, 1886; Scheunert & Trautmann, 1921; Scheunert, Krzywanek & Zimmermann, 1929; Denton, 1957*a*, *b*). The rate of secretion is reduced but is not annulled by acute or chronic denervation of the gland (Eckhard, 1867, 1893; Krinitsin, 1940; Coats, Denton, Goding & Wright, 1956), nor by the injection of atropine (Babichev, Perstnov & Kulesco, 1930; Coats *et al.* 1956).

Clark & Weiss (1952) were able to stimulate parotid secretion in anaesthetized sheep by brushing either the oesophagus or the cardiac region of the rumen; the sensory fibres concerned coursed in the cervical vagus nerves. On the other hand Coats *et al.* (1956) found that although chemical, electrical or mechanical stimulation of the mouths of sheep sometimes increased the rate of parotid secretion, such stimuli were ineffective when applied to the rumen.

The experiments described in this paper confirm and extend earlier work on the secretion of parotid saliva in sheep and define some of the reflex mechanisms which influence the rate of secretion. Observations were also made on the submaxillary glands of sheep and on the parotid glands of goats and calves. A preliminary account of some of these results has been given (Comline & Kay, 1955).

METHODS

Preparations. Nineteen experiments were undertaken on Welsh Mountain sheep 6–15 months of age. These were decerebrated in the manner described by Comline & Titchen (1951). After fasting for 12–48 hr, anaesthesia was induced with ethyl chloride and maintained with ether; the brain stem was transected in a plane passing through the anterior colliculi and the mammillary bodies, and anaesthesia was then discontinued.

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Other experiments were conducted on adult sheep and goats and on Jersey calves under chloralose anaesthesia. Chloralose (40–50 mg/kg body weight) was injected through a cannula inserted into the femoral vein after anaesthesia had been induced with ethyl chloride and maintained with ether.

Nerve stimulation. Perspex-shielded bipolar platinum electrodes were used for the stimulation of nerve trunks. The stimuli were delivered from a Palmer induction coil giving about 70 pairs of impulses per second, or from a neon tube stimulator.

In ruminants the secretory nerve to the parotid gland is derived from the buccal branch of the mandibular nerve and forms filaments which join the parotid duct on the surface of the masseter muscle and course beside the duct into the gland (Moussu, 1890). This nerve was called the parotid nerve by Moussu and will be referred to as such in this paper; it has also been called Moussu's nerve (Coats *et al.* 1956).

The parotid nerve was isolated and stimulated either below the masseter muscle, together with the buccal nerve, or in its course beside the parotid duct. Intracranial stimulation of nerves was carried out after removal of the medulla and mid-brain from preparations which had previously been decerebrated. After thoracotomy, the oesophageal branches of the vagus nerves were isolated and stimulated 2·5–5 cm anterior to the diaphragm; such sheep were ventilated with a 5% CO_2 + 95% O_2 gas mixture from a positive pressure pump.

Records. One or both parotid ducts were cannulated on the surface of the masseter muscle, care being taken to avoid damage to the parotid nerve. The rate of delivery of drops of saliva was recorded with a Thorp impulse counter. The volume of the drops varied with their rate of formation. For example, in one case in which the electrodes of the drop counter tube were 4 mm apart, it was found that the average volume of a drop of parotid saliva was 0·053 ml. at a flow of 1 ml./min, 0·057 ml. at 5 ml./min, and 0·064 ml. at 10 ml./min; the corresponding figures for the volumes of drops of blood were 0·046, 0·049, and 0·054 ml. In most experiments the rate of secretion did not exceed 5 ml./min.

Blood pressure was recorded from the femoral artery. At the end of most experiments the parotid glands were excised, freed from lymph glands and weighed.

Injections. Injections into the common carotid artery were made through fine polythene tubing inserted centrally via a thyroid or pharyngeal artery, and intravenous injections were given into a cannulated femoral vein. All doses are given as amount of drug per kilogram body weight. Acetylcholine chloride (Roche Products Ltd), atropine sulphate (British Drug Houses), Diamox (acetazolamide, 2-acetamido-1,3,4-thiadiazole-5-sulphonamide; Lederle), heparin (Roche Products Ltd), hexamethonium bromide (May & Baker) and pilocarpine nitrate (British Drug Houses) were each dissolved in 0·9% NaCl solution. Rogitine (phentolamine; Ciba) was injected as the solution supplied by the manufacturers.

RESULTS

Secretion of the denervated sheep parotid gland

A continuous secretion of parotid saliva was observed in all experiments. Section of the parotid nerve did not alter the rate of secretion in the majority of anaesthetized preparations, although in many decerebrate preparations the flow was somewhat diminished. The rate of secretion of a denervated parotid gland was generally between 0·5 and 1·6 ml./g parotid tissue/hr.

Isolation of the denervated parotid gland from all connexions with the preparation except the carotid artery and jugular vein did not further reduce its rate of secretion. In four cases such isolated glands were excised and perfused with blood from a heart–lung preparation of the sheep. These glands continued to secrete until the heart–lung system failed 1–4 hr later, but generally at a lower rate. In addition, the composition of the saliva changed at the onset of perfusion in that the concentrations of sodium and bicarbonate fell and those of potassium and chloride rose. The blood flow through the perfused glands was unusually high.

The intravenous injection of drugs blocking nervous transmission was no more effective in reducing parotid secretion than denervation of the gland. The injection of atropine sulphate 1 mg/kg or of hexamethonium bromide 2 mg/kg, which wholly extinguished reflex excitation of the parotid, did not reduce the rate of secretion of the denervated gland. Even as much atropine sulphate as 10 mg/kg was without effect. Injection of the adrenergic blocking agent Rogitine 1 mg/kg, which was sufficient to annul the effects on the parotid of stimulation of the peripheral end of the cervical sympathetic nerve (Kay, 1958*b*), did not influence the rate of secretion. In addition it was found that the injection of the carbonic anhydrase inhibitor Diamox 30 or 40 mg/kg into two anaesthetized sheep that had not been deprived of food did not affect either the rate of flow or the composition of the parotid saliva, although it caused a marked increase in urinary bicarbonate and base.

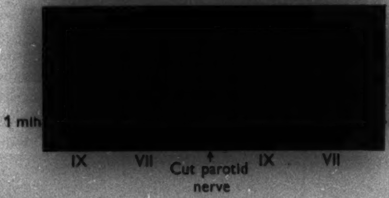

Fig. 1. Spinal sheep. The effects of intracranial stimulation of the peripheral ends of the glossopharyngeal (IX) and facial (VII) nerves on parotid secretion (induction coil, 12 cm). The responses disappeared after section of the parotid nerve. Records from above downwards: parotid saliva flow (drop counter returned to base line at 30 sec intervals); signal; time marker, 1 min.

Secretory innervation of the sheep parotid gland

Intracranial stimulation of the peripheral ends of cranial nerves gave similar results in three preparations. The rate of parotid secretion was increased by stimulation of the glossopharyngeal nerve and to a less extent by stimulation of the facial nerve (Fig. 1). Section of the parotid nerve or the injection of hexamethonium bromide abolished these responses. Stimulation of the peripheral ends of the trigeminal, vagus, accessory and hypoglossal nerves did not increase the rate of secretion.

Stimulation of the peripheral end of the buccal branch of the mandibular nerve produced a copious secretion of parotid saliva. This response was not reduced by injection of hexamethonium bromide 2 mg/kg which annulled the reflex excitation of the opposite gland. It appeared, therefore, that the parotid secretory filaments that course in the buccal nerve consist of post-ganglionic fibres. Histological examination supported this view; bundles of fine unmyelinated fibres were found within the buccal nerve, and such fibres formed the filaments that ran beside the parotid duct and its tributaries.

In some experiments the parotid was dissected out until the jugular vein carried only the venous blood flowing from the gland. After the injection of heparin this venous effluent was diverted through a cannula tied into the central end of the facial vein and recorded with a drop counter. Fig. 2 shows the effects of stimulating the peripheral end of the buccal nerve in such a preparation. It may be seen that the parotid blood flow was increased markedly and that the relative increase in salivary secretion was even greater. Reflex stimulation of the gland had similar effects.

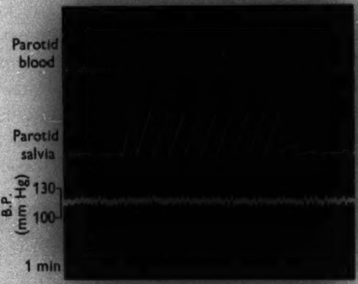

Fig. 2. Sheep; chloralose anaesthesia. The effects of stimulation of the peripheral end of the parotid nerve on the flow of parotid blood and parotid saliva (induction coil, 9·5 cm). Records from above downwards: parotid venous blood flow (drop counter returned to base line at 5 sec intervals); parotid saliva flow (drop counter returned to base line at 30 sec intervals); blood pressure; signal; time marker, 1 min.

Fig. 3 demonstrates that the intravenous injection of atropine sulphate 20 μg/kg caused a marked and equal reduction in both the secretion of saliva and the vasodilation produced either by stimulation of the buccal nerve or by the intracarotid injection of 10 μg of acetylcholine chloride. The responses were completely annulled 30 sec after a further injection of atropine sulphate 100 μg/kg.

Reflex stimulation of parotid secretion in sheep

Stimulation of the mouth. In decerebrate preparations either mechanical or chemical stimulation of the mouth increased the flow of parotid saliva. Rubbing a brush between the molar teeth and the tongue was the most effective stimulus (Fig. 4). The secretory response of the ipsilateral gland was much greater than that of the contralateral gland. Other areas of the mouth were usually much less sensitive in this respect; for instance, brushing between the molar teeth and the cheek (Fig. 4), or between the incisors, dental pad and

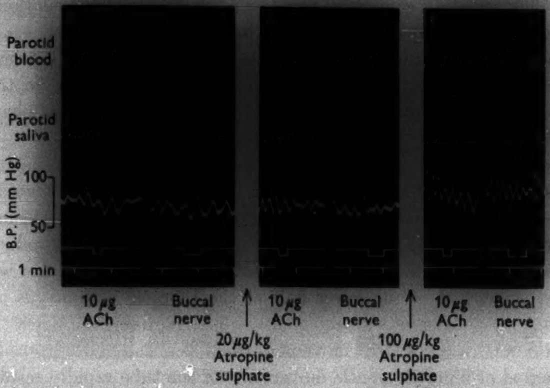

Fig. 3. Sheep; chloralose anaesthesia. The I.V. injection of atropine abolished both the secretory and the vascular responses of the parotid gland to the injection of 10 µg of acetylcholine chloride (ACh) into the ipsilateral carotid artery and to stimulation of the peripheral end of the buccal nerve (induction coil, 8 cm). Records from above downwards: parotid venous blood flow (drop counter returned to base line at 5 sec intervals), parotid saliva flow (drop counter returned to base line at 30 sec intervals); blood pressure; signal; time marker, 1 min.

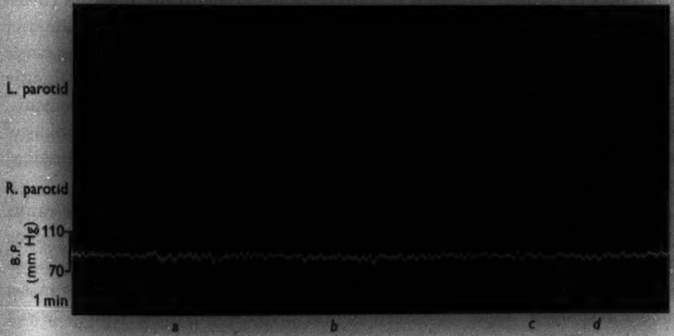

Fig. 4. Decerebrate sheep. Stimulation of the buccal epithelium. Records from above downwards: left and right parotid saliva flow (drop counters returned to base line at 1 min intervals); blood pressure; signal; time marker, 1 min.

Rubbing a test-tube brush between the tongue and molar teeth, on the left (*a*) and right (*b*) sides increased parotid secretion, especially ipsilaterally, but brushing similarly between the cheeks and molar teeth on the left (*c*) and right (*d*) sides had no effect.

lips, had little or no influence on parotid secretion. The introduction of 20 ml. of 0·5 M acetic acid into the mouth increased the flow of parotid saliva in some experiments but not in all; the same volume of tap water, of 0·5 M-NaCl, or of 0·1 N-HCl produced no response.

The reflex secretion caused by mechanical or chemical stimulation of the mouth was abolished by cutting both the lingual and glossopharyngeal nerves or the parotid nerve, or by the intravenous injection of atropine. Stimulation of the central end of the lingual nerve usually caused a two- to threefold increase in parotid secretion. The effect of stimulation of the glossopharyngeal nerve was less regular.

The oesophagus. In two preparations the oesophagus was transected and cannulated in the neck, and the upper and lower parts were stimulated with a bottle brush. Stimulation of the upper cervical oesophagus had little influence on parotid secretion but the flow of saliva was doubled by the introduction of the brush into the thoracic oesophagus. A much greater response was produced by distension of the lower half of the thoracic oesophagus and the abdominal oesophagus with a rubber tube 2·5 cm in diameter. When the tube was held in a position without movement the flow of saliva diminished slowly over a period of minutes; gentle movements of the tube rapidly restored the flow to its former high rate.

The rumen and reticulum. In twelve decerebrate or anaesthetized preparations the dorsal sac of the rumen was incised and the contents were removed to allow manual stimulation of the epithelium of the forestomach. Light stroking of the cardiac orifice was the most effective stimulus to parotid secretion. The initial rate of parotid secretion in a decerebrate preparation, after its rumen had been emptied, is shown in Fig. 5*a*. A hand was then placed in the anterior dorsal sac of the rumen, increasing the salivary flow by about three times (Fig. 5*b*, first minute). The mucosa of the anterior dorsal sac was slightly stretched and was gently rubbed against the hand by respiratory movements. Light stroking of the cardia with a finger-tip then caused a further doubling of parotid secretion after a delay of about 5 sec. All these responses were annulled by injection of atropine (Fig. 5*c*).

Responses that were almost as great as those elicited by stroking the cardia were obtained by light stroking of the reticulo-omasal orifice or by stretching this orifice or the cardia with a finger. Stroking the lips of the oesophageal groove, the reticulo-rumen fold or the epithelium of the reticulum gave rather smaller responses. Light sustained digital pressure on these regions was a much less effective stimulus than touch or stretch. Parotid secretion was not increased by mechanical stimulation of the walls of the ventral sac or of the posterior part of the dorsal sac of the rumen.

Parotid secretion was also increased in four decerebrate preparations by the introduction into the empty washed rumen of 100 ml. of each of the following acids: 0·5 M-solutions of acetic acid, propionic acid, *n*- or *iso*-butyric acid, lactic acid or 0·1 N-HCl. Similar responses were obtained with each of these acids. The responses began after a delay of about 1 min and lasted for about 5 min; the peak rate of secretion was two to four times greater than the resting flow. The introduction of 100 ml. of tap water, 0·9% NaCl, 1·15% KCl, or 0·5 M sodium acetate produced no response.

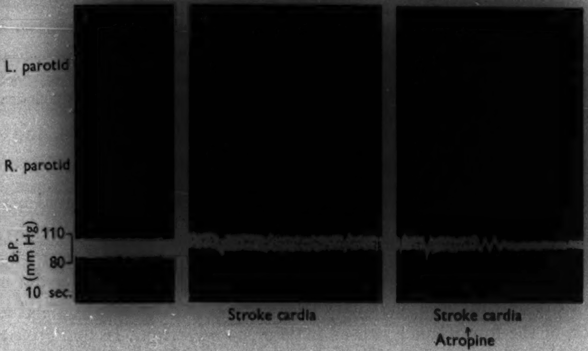

Fig. 5. Decerebrate sheep. Parotid secretion in response to stimulation of the cardia. Records from above downwards: left and right parotid saliva flow (drop counters returned to base line at 1 min intervals in *a*, and at 30 sec intervals in *b* and *c*); blood pressure; signal; time marker, 10 sec. *a*, Rumen empty; *b*, hand in rumen; cardia stroked with a finger-tip; *c*, cardia stroked with a finger-tip, and the response annulled by the I.V. injection of atropine sulphate 0·5 mg/kg.

The reflex secretion by the parotid gland during mechanical stimulation of structures in the stomach was completely abolished by section of either the thoracic or the cervical vagus nerves. In the thorax the distribution of the sensory fibres concerned between the dorsal and the ventral oesophageal branches of the vagus nerves appeared to be variable. These nerves were cut 2·5–5 cm anterior to the diaphragm and their central ends stimulated in five preparations. In one, stimulation of the central end of the dorsal branch caused a tenfold increase in parotid secretion after a delay of about 5 sec (Fig. 6); stimulation of the ventral branch had no effect. In the remaining four preparations parotid responses could be obtained from either branch, but only in one preparation was a response obtained from the ventral branch as great as that obtained from the dorsal.

After section of one cervical vagus nerve, the reflex response of the ipsilateral parotid gland to mechanical stimulation of the forestomach was much reduced; that of the contralateral gland was only lessened to a slight extent. Similarly, stimulation of the central end of one cervical vagus caused the ipsilateral parotid to secrete much more rapidly than the contralateral gland.

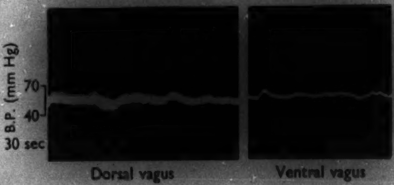

Fig. 6. Decerebrate sheep. Stimulation of the branches of the thoracic vagus nerve (induction coil, 13 cm). Records from above downwards: parotid saliva flow (drop counter returned to base line at 30 sec intervals); blood pressure; signal; time marker, 30 sec. *a*, stimulation of the dorsal oesophageal vagus; *b*, stimulation of the ventral oesophageal vagus.

The submaxillary gland of the sheep

This gland was found to be innervated by a branch of the lingual nerve which enters the gland with the submaxillary duct; this arrangement is similar to that found in other species. In most anaesthetized preparations a very slow continuous flow of submaxillary saliva occurred but this did not exceed a rate of 0·01 ml./g submaxillary tissue/hr. In decerebrate preparations stimulation of the forestomach caused little or no secretion. In one preparation stimulation of the central end of the cervical vagus nerve produced a flow of saliva from the ipsilateral submaxillary gland of 0·15 ml./g submaxillary tissue/hr; this was less than one tenth of the flow elicited by stimulation of the central end of the ipsilateral lingual nerve.

The parotid glands of goats and calves

No differences were found between the innervation and responses of the parotid glands of goats and those described for sheep. The continuous secretion of the denervated gland was observed and reflex secretion was caused by mechanical stimulation of the sensitive regions of the mouth, oesophagus and forestomach.

The innervation of the parotid gland of the calf was also similar to that described in the sheep. In calves less than 1 month old there was no continuous secretion of parotid saliva and stimulation of the peripheral end of the cut parotid nerve or the intravenous injection of pilocarpine elicited little response from the gland. By the age of 3 months the continuous parotid secretion was apparent and persisted after denervation of the gland and a copious flow of saliva was caused by stimulation of the parotid nerve. In these older calves, light stroking of the cardia, the oesophageal groove or the walls of the reticulum produced a rapid reflex secretion of parotid saliva. The responses were abolished by section of the parotid nerve or by injection of atropine.

DISCUSSION

The secretion of the denervated parotid gland. It was shown by Eckhard (1867, 1893) and confirmed by Krinitsin (1940) and by Coats *et al.* (1956) that the parotid gland of the sheep continues to secrete following denervation. The experiments reported in this paper give further support to this observation. In addition to its resistance to atropine (Babichev *et al.* 1930; Coats *et al.* 1956) the secretion of the denervated gland is unaffected by the injection of hexamethonium, of the adrenergic blocking agent Rogitine and of the carbonic anhydrase inhibitor Diamox. It differs from the 'paralytic' secretion of the denervated submaxillary gland (Bernard, 1864) in that it is fully present from the time of denervation and not only after the lapse of several days, during which time the submaxillary gland becomes supersensitive to excitatory agents (Emmelin, 1952). Atropine-insensitive secretion by denervated salivary glands is not unknown, for it has been carefully studied by Emmelin (1953) in excised sublingual glands of the cat.

Krinitsin (1940) found that the secretion of the denervated ruminant parotid progressively diminishes during prolonged starvation and suggested that this secretion is not due to some property of the gland itself, as claimed by Eckhard (1893), but is stimulated by absorbed products of digestion. Such a theory does not explain the secretion of the isolated and perfused parotid gland, first demonstrated by Coats *et al.* (1956), and further experiments are required to establish the cause of this secretion.

It is unlikely that the continuous secretion contributes more than a small fraction of the total volume of parotid secretion. In twenty-three of the sheep used in the present experiments, whose average weight was 34 kg, the average weight of a single parotid gland was 13 g. The resting secretion of a 13 g parotid, assuming a rate of 0·5–1·6 ml./g parotid tissue/hr, would amount to about 150–500 ml. of saliva/24 hr, whereas in conscious sheep a single parotid is reported to secrete 1–4 l. saliva/24 hr (Denton, 1957*b*).

Two aspects of the flow of blood through the sheep's parotid gland deserve attention. The first is that stimulation of the buccal nerve does not increase the parotid blood flow to as great an extent as the rate of secretion of saliva. This suggests that at rapid rates of secretion there may be a relative reduction in the amount of plasma electrolytes that are available for the formation of a given volume of saliva. This factor may contribute to the depression of the concentrations of potassium and phosphate in the saliva that is observed as the rate of secretion is increased (Coats & Wright, 1957). The second point is that the nervously excited secretion and the attendant vasodilation in the sheep's parotid are equally and delicately sensitive to blockage by atropine. In the submaxillary gland it has long been known that glandular vasodilation is much less sensitive to atropine than is salivary secretion (Heidenhain, 1872).

No explanation can be offered for the different reaction of the sheep's parotid gland.

Reflex secretion. The present experiments have defined many of the reflex arcs controlling parotid secretion. In all cases the efferent limb consists of preganglionic fibres that leave the brain with the facial and glossopharyngeal nerves and of post-ganglionic fibres that initially course with the buccal nerve and subsequently reach the gland as filaments that accompany the parotid duct. The position of the synapses between these fibres has not been studied, but it is probably in the otic ganglion, as in other species (Shute, 1956).

The afferent limbs of the reflex arcs have been established as fibres from the stomach which course in the vagus nerves and as fibres from the mouth distributed between the facial and glossopharyngeal nerves. The methods employed in the present experiments do not permit an exact definition of the nature of the stimuli to which the receptors concerned in parotid secretion respond. Striking responses were associated with brushing or light stroking of certain regions of the mouth and of the cardia, the reticulo-omasal orifice, the lips of the oesophageal groove, the reticulo-rumen fold and nearby regions. Such stimuli were presumably largely tactile in nature, but undoubtedly the stroking would have dragged on the epithelium and compressed the musculature to a small extent. In addition, stretch of the terminal oesophagus, cardia and reticulo-omasal orifice proved little less effective, and in this form of stimulation stretch and compression of the epithelium and musculature would have predominated. Stretch of the reticulo-rumen fold (Comline & Titchen, 1957) and distension of the oesophagus with gas (Kay & Phillipson, 1957) have also been found to increase greatly the flow of parotid saliva in decerebrate or anaesthetized sheep.

In decerebrate preparations of sheep, goats and calves Titchen (1958) was able to initiate or augment contractions of the reticulum by stretch of the reticulum, omasal canal and abomasum and by tactile stimulation of the lower thoracic oesophagus and abomasum. Ash & Kay (1957) found that in conscious sheep stretch or tactile stimulation of the lower thoracic oesophagus, cardia and reticulo-omasal orifice both excited parotid secretion and had variable effects on reticulum motility. The evidence suggests that both stretch and tactile receptors occur in the ruminant forestomach and that each group of receptors may influence both parotid secretion and reticulum motility. The stretch receptors concerned may be similar to those studied in the goat by Iggo (1955) who recorded afferent volleys in the vagus nerve from tension receptors in the reticulum. Nervous structures that may account for the sensitivity to touch are those found by Hill (1957) in the subepithelial connective tissue of the forestomach of the goat; fibrils from these structures penetrate into the keratinized layer of the epithelium.

The reflex parotid secretion caused by stimulation of the buccal epithelium on one side, or of the central end of one cervical vagus nerve, was largely confined to the ipsilateral gland. This suggests that the afferent connexions of the centres regulating parotid secretion are predominantly unilateral and that there is little communication between the centres on one side of the brain stem with those on the other. Similar conclusions were reached concerning the salivary centres of other species by Chatfield (1941) and by Magoun & Beaton (1942) as a result of direct stimulation of the medulla.

The facility with which ruminant parotid secretion is excited by mechanical stimulation of the forestomach is not equalled in monogastric species, in which the receptors concerned with salivary secretion are largely confined to the mouth. Even in the latter group gastric receptors may have some influence on salivary secretion, for in the dog Oehl (1864) was able to excite reflex secretion of the submaxillary gland by introducing mustard, alcohol, etc., into the stomach, or by stimulation of the central end of the vagus nerve until nausea was apparent. In ruminants the parotid saliva plays an important part in ruminal digestion, in addition to aiding mastication, and mechanisms that relate the activity of the parotid gland to the physical conditions in the forestomach are clearly of value.

Krasuski, Krynskaya & Kotlyarevskaya (1940) found that a variety of organic and mineral acids and salts would influence the rate of parotid secretion when introduced into the mouth or rumen, but in general the effects were small. The concentration and pH of the organic acids used to stimulate the rumen in the present experiments were well outside the values found in conscious sheep (Phillipson, 1942). Thus it is not yet possible to say whether parotid secretion is reflexly influenced by the chemical composition of the rumen contents of sheep under normal conditions.

The parotid glands of goats and calves. The studies on the parotid glands of goats and three-month-old calves have shown that they closely resemble the parotid gland of the sheep in their innervation and reflex control.

In calves less than a month old the parotid gland was able to produce little or no saliva. This gland has been found to be similarly immature in young goats (Kay, 1958*a*). The absence of parotid secretion in young calves has been noted and investigated by Krinitsin (1940) who suggested that the development of parotid secretion is related to the onset of ruminal digestion. However this may be, the immaturity of the parotid gland is probably of little consequence before mastication of solid food and ruminal digestion begin.

SUMMARY

1. The secretion of parotid saliva has been examined in decerebrate and anaesthetized sheep and in anaesthetized goats and calves.
2. The secretory innervation consisted of preganglionic fibres in the glosso-

pharyngeal and facial nerves. The post-ganglionic fibres were distributed to the gland in the parotid nerve, a branch of the buccal nerve.

3. Parotid secretion continued after section of the parotid nerve and following the injection of atropine, hexamethonium, Rogitine and Diamox.

4. Both the secretory and the vasodilator responses to stimulation of the parotid nerve were annulled by atropine.

5. Reflex parotid secretion was caused by: (*a*) mechanical stimulation of the mouth, the lower thoracic oesophagus, the cardia, the reticulo-omasal orifice and to a less extent the lips of the oesophageal groove, the reticulo-rumen fold and the epithelium of the reticulum; or (*b*) the introduction of acid into the mouth or rumen.

6. The afferent fibres concerned coursed from the mouth with the lingual and glossopharyngeal nerves and from the forestomach with the vagus nerves.

7. Little or no parotid secretion was found in calves less than one month old.

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THE EFFECTS OF STIMULATION OF THE SYMPATHETIC NERVE AND OF ADRENALINE ON THE FLOW OF PAROTID SALIVA IN SHEEP

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Stimulation of the cervical sympathetic nerve or the injection of adrenaline has been shown to produce a flow of saliva in many species. This has been attributed either to the expulsion of pre-formed saliva or to the secretion of saliva, or to some combination of these effects, but it has been recognized that the vasoconstriction caused by sympathetic stimulation or adrenaline may weaken an existing secretion. This subject was fully reviewed and discussed by Babkin (1950).

In the cat and dog the alterations in salivary flow caused by sympathetic stimulation or by adrenaline injection are usually small, both absolutely and relative to the corresponding parasympathetic effects, and are difficult to interpret. In the present experiments the responses to sympathetic stimulation and adrenaline have been studied in the parotid gland of the sheep. The ruminant parotid differs from the salivary glands of other species in that it produces a continuous flow of non-viscous saliva, even when denervated (Eckhard, 1867). Superimposed upon this resting flow the effects of sympathetic stimulation and adrenaline may readily be demonstrated and studied.

Stimulation of the sheep's cervical sympathetic nerve causes a rapid increase in the flow of parotid saliva. This response was first reported by von Wittich (1866), who considered it to be a secretory effect. Eckhard (1867, 1869) showed that the phase of increased flow was only transitory and was followed by a compensatory pause, and attributed the response to an expulsion of saliva from the gland. This observation has been confirmed and extended in the present experiments. Adrenaline, on the other hand, not only causes a small expulsion of saliva by a direct action on the gland, but also elicits a prolonged increase in the rate of secretion by a reflex action.

Preliminary accounts of this work have been published (Kay, 1954, 1955). Coats, Denton, Goding & Wright (1956) have also described experiments which support the conclusion reached by Eckhard. In addition, these authors have shown that sympathetic stimulation, the injection of noradrenaline and graded constriction of the carotid artery each reduce the flow of saliva resulting from stimulation of the parotid nerve.

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METHODS

Preparations. Fourteen experiments devoted primarily to examination of the response to stimulation of the cervical sympathetic nerve were carried out on adult sheep, mostly Suffolks, weighing 30–70 kg; sheep of this size gave parotid responses that were sufficiently large for convenient measurement. In five experiments in which parotid blood flow was measured Welsh Mountain sheep weighing 10–20 kg were used. The twelve sheep in which the influence of adrenaline was studied were of either breed.

The animals were deprived of food and bedding for 24–48 hr before the experiments, but had free access to water. Anaesthesia was induced with ethyl chloride and maintained with ether while chloralose (40–50 mg/kg) was injected into a cannulated femoral vein.

Records. Drop records of the flow of saliva and of venous blood from the parotid gland were obtained by methods described in the preceding paper (Kay, 1958). Flow of saliva was also recorded with a water manometer in some experiments. Although these methods of recording were sufficiently accurate for the purposes of these experiments, it should be noted that neither gives a strictly quantitative measure of the rate of secretion of saliva. The size of the drops increases slightly as the rate of flow is raised, while an increase of pressure in a recording manometer was found to dilate the ducts and alveoli of the parotid gland, so that a fraction of the newly secreted saliva was accommodated within the gland.

Blood pressure was recorded from the femoral artery. The parotid nerve was isolated and stimulated either beneath the masseter muscle, together with the buccal nerve, or in its course beside the parotid duct. The cervical sympathetic nerve was exposed in the mid-cervical region. If at this point it was partially or wholly fused with the vagus, the common trunk was followed towards either the inferior or the superior cervical ganglion until the nerves separated and a sufficient length of the sympathetic could be isolated.

Injections. Intravenous injections were given into a cannulated femoral vein. Injections into the common carotid artery were made through fine polythene tubing inserted centrally via a thyroid or pharyngeal artery. Injections directly into the coeliac artery were given through a fine polythene tube inserted centrally into the splenic artery after removal of the spleen, and those into the aorta through tubing inserted centrally through a femoral artery. In the latter case heparin 1000 u./kg body wt. was administered to prevent blood coagulation. The positions of the tips of the polythene tubes within the coeliac artery and the aorta were determined post-mortem.

L-Adrenaline base (British Drug Houses) was dissolved to make 200 μg/ml. solution in 0·9% NaCl solution acidified to pH 3 with HCl. Atropine sulphate (British Drug Houses), hexamethonium bromide (May & Baker), heparin (Roche Products Ltd.) and nicotine hydrogen tartrate (British Drug Houses) were dissolved in 0·9% NaCl. Oxytocin (Pitocin; Parke, Davis), vasopressin (Pitressin; Parke, Davis) and Rogitine (phentolamine; Ciba) were injected as the solutions supplied by the manufacturers.

RESULTS

The resting flow of saliva from the parotid gland was continuous and regular in all the experiments except one. In the single exception the parotid saliva flowed intermittently in sudden gushes, separated by 1–2 min of relative quiescence.

Sympathetic stimulation. Stimulation of the peripheral end of the cervical sympathetic nerve for periods of up to 5 sec caused a rapid increase in the flow of parotid saliva from the ipsilateral gland, followed by complete cessation. The increase began within 1 sec of applying the stimulus. The duration of the pause which followed was proportional to the size of the initial increase in flow, so that it compensated almost exactly for this increased flow. The flow from the contralateral gland was not affected. These effects are shown in Fig. 1. In the experiment from which this record was obtained the parotid flow before stimulation was about 10 drops/min. The average flow during the period of increased flow evoked by the stimulus and during the succeeding compensatory pause was 11 drops/min.

These responses were also recorded with a water manometer (Fig. 2). In this tracing the resting flow is recorded as a steady rise in the level of fluid in the manometer. Stimulation of the peripheral end of the cervical sympathetic nerve caused an abrupt increase in the flow of saliva into the manometer. After the stimulus ceased most of the additional saliva flowed back into the gland so that the tracing returned to the level which would have been expected if the stimulus had not been delivered.

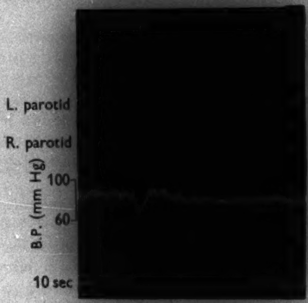

Fig. 1

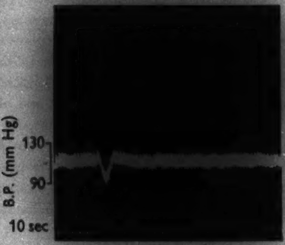

Fig. 2

Fig. 1. The effect of stimulation of the peripheral end of the left cervical sympathetic nerve for 5 sec on the flow of parotid saliva. Records from above downwards: flows of saliva from left and from right parotids recorded with drop counters, the counters being returned to the base line at 1 min intervals; blood pressure; signal; time in 10 sec intervals.

Fig. 2. The effect of stimulation of the peripheral end of the cervical sympathetic nerve for 3 sec on the flow of parotid saliva. Records from above downwards: parotid flow recorded with a water manometer; blood pressure; signal; time in 10 sec intervals.

These results suggested that a short period of stimulation of the sympathetic nerve causes pre-formed saliva to be expelled from the parotid but does not appreciably affect the rate of secretion. This conclusion was supported by the effect of repeated periods of stimulation each of 5 sec duration, delivered at intervals of 1 min (Fig. 3). The response to the first stimulus is similar to that shown in Fig. 1. During the succeeding periods of stimulation the number of drops of saliva recorded each minute diminished until on the fourth stimulus it was equal to that secreted before stimulation. The pause in the flow which followed the last stimulus compensated almost exactly for the extra volumes expelled during the first three periods of stimulation.

Fig. 3. The effect of stimulation of the peripheral end of the right cervical sympathetic nerve for 5 sec, repeated ten times at 1 min intervals, on the flow of parotid saliva. Records from above downwards: drop records of right and of left parotid flows, the counters being returned to the base line at 1 min intervals; blood pressure; signal; time in 10 sec intervals.

Prolonged stimulation of the cervical sympathetic nerve gave essentially similar results. For example, in one experiment the nerve was stimulated continuously for 5 min. During the first 30 sec the flow increased in a manner similar to that shown in Fig. 1, but subsequently dwindled to less than the resting flow recorded before stimulation. On cessation of the stimulus the flow stopped for 2 min before the resting flow reappeared. Again, the average rate of flow during the period of stimulation and recovery was the same as that recorded before stimulation. Very long periods of sympathetic stimulation somewhat reduced the rate of flow. For example, it was found that a flow of 1·6 ml./g parotid tissue/hr during a control period was reduced to one of 0·9 ml./g parotid tissue/hr during 30 min of continuous stimulation.

Individual variation was found in the volume of saliva expelled as a result of sympathetic stimulation. Figs. 1–3 show responses that were larger than usual. In some sheep the parotid response was very small, the compensatory pause being reduced to less than a minute, although pupillary dilation and exophthalmos occurred normally. Nevertheless, the form of the response was the same in all preparations and no evidence was found of any appreciable increase in the rate of secretion.

The contractile tissue. Sections of sheep parotid gland stained with haematoxylin and eosin were examined to determine whether smooth muscle present in the gland could account for the expulsion of saliva. The only smooth muscle detected was that associated with the blood vessels. As was found by Babkin & MacKay (1930), neither the main excretory duct nor its tributaries possessed a muscular coat. However, Silver (1954) had reported the presence of large numbers of myo-epithelial cells in the sheep parotid gland, and in order to gain some indication of whether this myo-epithelium was responsible for the sympathetic expulsion of saliva a study was made of the effects on salivary flow of the intravenous injection of oxytocin and vasopressin.

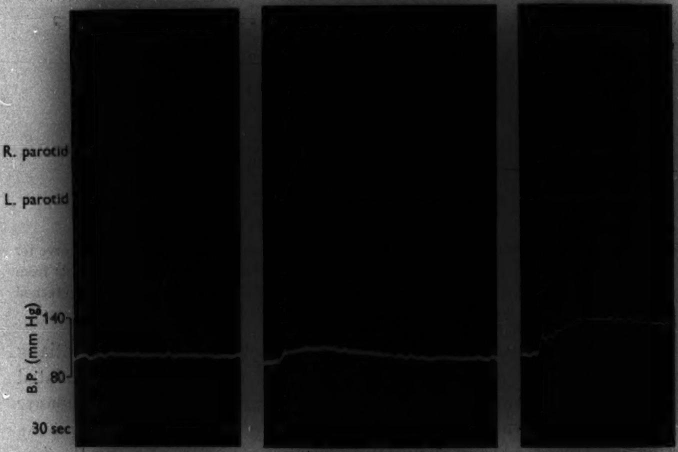

Fig. 4. The action of oxytocin and vasopressin on the flow of parotid saliva. Records from above downwards: right parotid flow recorded with a water manometer; left parotid flow recorded with a drop counter returned to base line at 1 min intervals; blood pressure; signal; time in 30 sec intervals. *a*, I.V. injection of oxytocin 20 m-u./kg; *b*, I.V. injection of oxytocin 100 m-u./kg; *c*, I.V. injection of vasopressin 100 m-u./kg.

Fig. 4 illustrates such an experiment. The flow of saliva was recorded from one parotid with a water manometer and from the opposite gland with a drop counter. The intravenous injection of oxytocin 20 m-u./kg caused a small expulsion of saliva followed by a compensatory pause. With this dose there was no change in the blood pressure. A larger dose of oxytocin, 100 m-u./kg, produced a more substantial expulsion of saliva and a slight rise in the blood pressure. The injection of vasopressin 100 m-u./kg caused a greater rise in blood pressure but did not affect salivary flow.

The innervation of the contractile tissue by sympathetic nerve fibres was studied further by the use of drugs. The intravenous injection of hexamethonium bromide 2 mg/kg almost wholly annulled the response to stimulation of the sympathetic nerve at a point 5 cm caudal to the superior cervical ganglion. The application of 5% nicotine tartrate solution to this ganglion had a similar effect. The intravenous injection of Rogitine 1 mg/kg also annulled the response to sympathetic stimulation (Fig. 6).

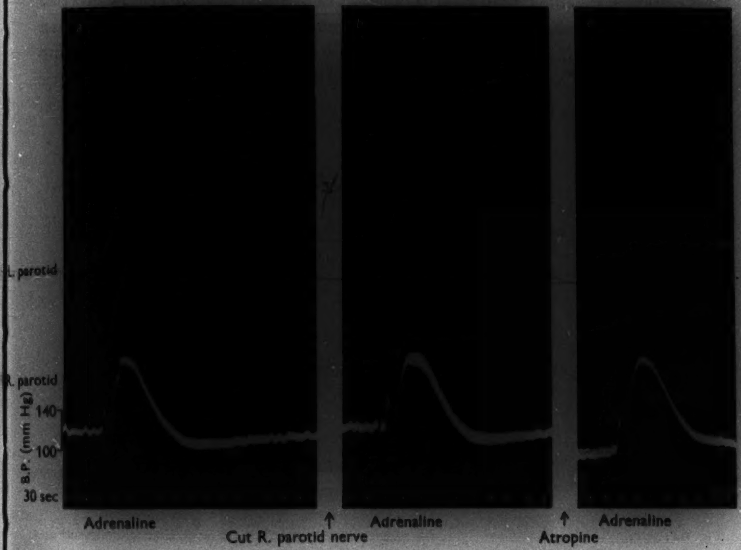

Fig. 5. The influence of adrenaline on the flow of parotid saliva. Records from above downwards: flow of saliva from left and from right parotids recorded with water manometers; blood pressure; signal; time in 30 sec intervals. *a*, I.V. injection of adrenaline 3 μg/kg; the right parotid manometer was emptied after 5 min to prevent the tracing leaving the paper. *b*, I.V. injection of adrenaline 3 μg/kg after section of the right parotid nerve. *c*, I.V. injection of adrenaline 3 μg/kg after the injection of atropine sulphate 1 mg/kg.

These results and the histological findings of Silver (1954) indicate that the tissue responsible for the expulsion of saliva from the sheep's parotid is myoepithelium innervated by post-ganglionic adrenergic sympathetic nerve fibres. The influence of adrenaline on the flow of parotid saliva was therefore examined.

Adrenaline. The changes in the flow of parotid saliva that followed the injection of adrenaline were more complex than those that followed stimulation of the sympathetic nerve supply. The two phases of the response could be distinguished most easily when recorded with a water manometer. Fig. 5 illustrates the effect of injecting adrenaline 3 μg/kg intravenously. 15–20 sec after the injection there was a small but rapid increase in flow; 5–10 sec later this was swamped by the appearance of a second and much greater acceleration of the flow which was maintained for several minutes. Similar results were obtained in the seven other preparations that were tested, using doses of adrenaline of 2–5 μg/kg, although the size of the response was variable. Generally the response declined gradually to reach the resting rate of secretion after 5–10 min, but in one case the flow of saliva remained augmented for more than 20 min. This second response was eliminated on one side (Fig. 5*b*) by cutting the buccal branch of the mandibular nerve, which carries the parasympathetic secretory fibres to the gland, and on the other side (Fig. 5*c*) by the injection of atropine.

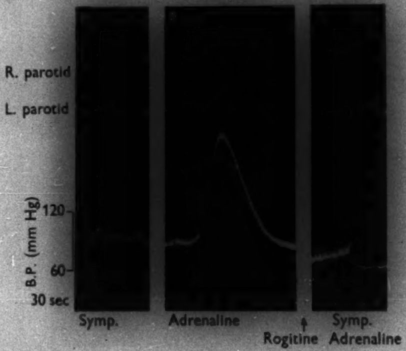

Fig. 6. The effect of Rogitine on the parotid responses to sympathetic stimulation and adrenaline. Records from above downwards: right parotid flow recorded with a water manometer; left parotid flow recorded with a drop counter returned to base line at 1 min intervals; blood pressure; signal; time in 30 sec intervals. *a*, The response to stimulation of the peripheral end of the right cervical sympathetic nerve (symp.) for 3 sec. *b*, The response to the I.V. injection of adrenaline 3 μg/kg, following an injection of atropine sulphate 0·5 mg/kg. *c*, A repetition of sympathetic stimulation (first signal) and adrenaline injection (second signal) 4 min after an injection of Rogitine 1 mg/kg.

After the injection of atropine the effect of an intravenous injection of adrenaline was similar to that of stimulation of the cervical sympathetic nerve; that is, there was a transient increase in the flow of saliva followed by a compensatory pause (Figs. 5*c*, 6*b*). The expulsion of saliva produced by the injection of adrenaline, like that caused by sympathetic stimulation, was annulled by the injection of Rogitine 1 mg/kg (Fig. 6).

The secretory action of adrenaline was largely abolished by section of the dorsal branch of the thoracic vagus nerve 2·5 cm anterior to the diaphragm. The reflex parotid secretion produced in two preparations by prolonged stimulation of the central end of this branch was not increased by the intravenous injection of adrenaline.

The site of action of adrenaline was examined further in five preparations by the injection of adrenaline 3 μg/kg into the abdominal aorta at different levels (Fig. 7). This dose produced a marked acceleration of parotid secretion, with a delay of 10–20 sec, when it was injected into the aorta just anterior to the origin of the coeliac and superior mesenteric arteries (Fig. 7*b*). It had a very much smaller effect with a delay of about 1 min when given just posterior to these arteries (Fig. 7*c*), and had no action when given 7 cm posterior to them (Fig. 7*d*). An increase in parotid secretion was obtained in two preparations about 10 sec after the injection of adrenaline 3 μg/kg directly into the coeliac artery via the splenic artery.

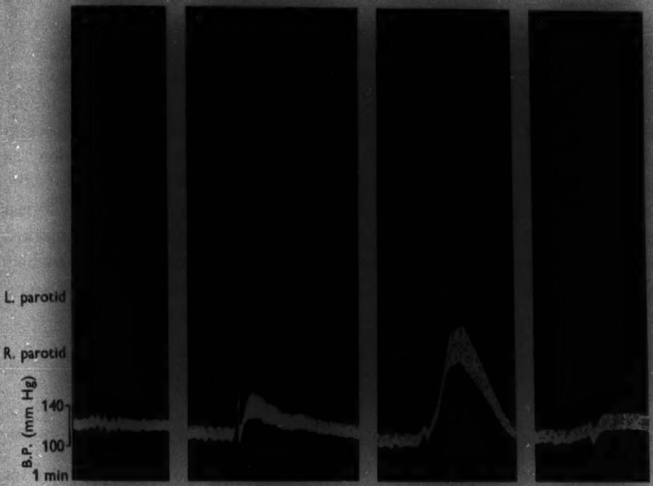

Fig. 7. The secretory response of the parotid to injections of adrenaline into the aorta. Records from above downwards: the flow of saliva from left and from right parotids recorded with water manometers; blood pressure; signal; time in 1 min intervals. *a*, Control injection of 1 ml. of acid saline given 10 cm anterior to the coeliac and superior mesenteric arteries; *b*, adrenaline 3 μg/kg injected 3 cm anterior to these arteries; *c*, adrenaline 3 μg/kg injected 2 cm posterior to these arteries; *d*, adrenaline 3 μg/kg injected 7 cm posterior to these arteries.

In two experiments a balloon was placed in the reticulum and in a third in the abomasum, and manometric records were taken of the movements of these organs. About 15 sec after the injection of adrenaline 3 μg/kg into the aorta anterior to the coeliac artery the reticulum contracted, returning to its initial relaxed state within 45 sec of the injection. The continuous movements of the abomasum were somewhat depressed for 2 min after a similar injection. In each experiment parotid secretion began to accelerate within 20 sec of the injection, reached a peak flow 1–2 min later and remained augmented for a further 3 min.

When adrenaline was injected repeatedly the secretory response diminished progressively and eventually disappeared. The application for 1 min of an effective stimulus to parotid secretion, such as distension of the oesophagus and cardia with a rubber tube inserted through the mouth (Kay, 1958), would then largely restore the response to further injections of adrenaline.

A third effect of adrenaline was to cause vasoconstriction in the gland. Injected into the carotid artery, adrenaline in doses of 1–3 μg/kg profoundly reduced the blood flow through an isolated denervated parotid for as long as 5 min. Injected intravenously such doses had little direct influence on the blood flow, which increased in parallel with the rise in blood pressure.

Diminution of parotid blood flow occurred during stimulation of the cervical sympathetic nerve. The vasoconstriction that resulted abolished the marked vasodilation produced by stimulation of the buccal nerve, and after a short delay was followed by a corresponding reduction in the rate of parotid secretion. The results of one such experiment are given below:

Stimulation of buccal nerve			←	—	—	—	—	—	—	—	—	→		
Stimulation of sympathetic nerve							↔							
Saliva (drops/30 sec)	2	2	27	19	17	14	13	6	9	13	12	12	5	2
Blood (drops/30 sec)	24	23	101	87	81	62	33	22	53	58	54	50	29	20

The responses seen in Figs. 8 and 9 may be attributed to the vasoconstrictor action of adrenaline. In Fig. 8*a* is shown the normal secretory response to stimulation of the peripheral end of the buccal nerve. Adrenaline 2 μg/kg was then injected into the ipsilateral carotid artery; 3 min later (Fig. 8*b*) the secretory response was much diminished, although after a further 20 min (Fig. 8*c*) it had largely recovered.

Figure 9 shows the responses of normally innervated parotids to an injection of adrenaline 2 μg/kg into one carotid artery. Within 2 sec the flow of saliva from the ipsilateral parotid suddenly increased and subsequently reversed in a manner typical of expulsion. The contralateral gland was not affected during this period, but after 1 min it gave a secretory response similar to that following the intravenous injection of adrenaline. At the peak of this response saliva began to flow from the ipsilateral gland also, but, as with the secretory response shown in Fig. 8*b*, this flow was not sustained.

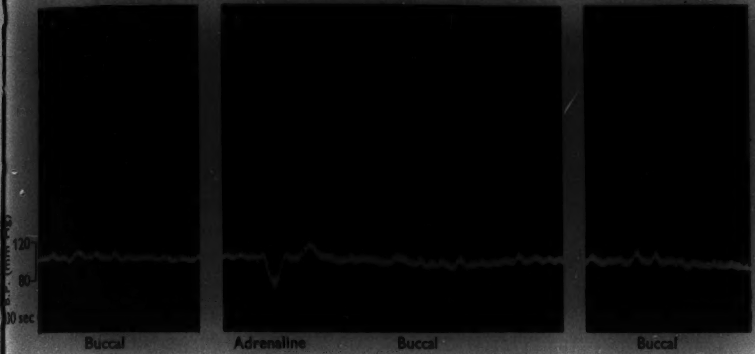

Fig. 8. The weakening of the secretory response to stimulation of the buccal nerve by injection of adrenaline into the carotid artery. Records from above downwards: parotid flow recorded with a water manometer; blood pressure; signal; time in 30 sec intervals. *a*, Control response to stimulation of the peripheral end of the buccal nerve for 1 min; *b*, adrenaline 2 μg/kg was injected into the ipsilateral carotid artery at the first signal, and the buccal nerve was stimulated 3 min later (second signal); *c*, stimulation of the buccal nerve 20 min later.

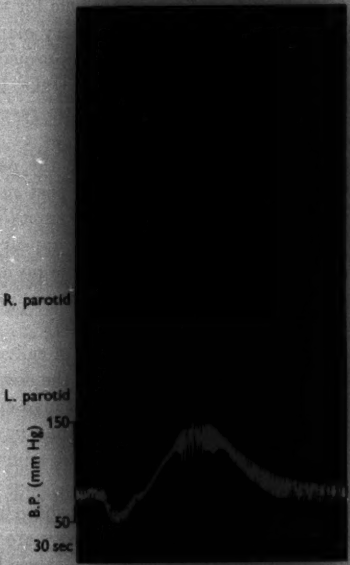

Fig. 9. The action of an injection of adrenaline 2 μg/kg into the left carotid artery. Records from above downwards: the flow of saliva from right and left parotids recorded with water manometers; blood pressure; signal; time in 30 sec intervals.

DISCUSSION

The experimental evidence given in this paper suggests strongly that the increase in the flow of parotid saliva of the sheep which occurs during stimulation of the cervical sympathetic nerve is due to an expulsion of pre-formed saliva. This conclusion is based largely on the observation that after the stimulus ceased the flow was interrupted to an extent that compensated almost exactly for the increased flow during stimulation. In addition, it may be noted that the onset of the response to sympathetic stimulation was more abrupt than that of the response to stimulation of the parotid nerve. If any change in the rate of secretion occurred it was so small as to be masked by the expulsion. These results confirm and extend the observations of Eckhard (1867, 1869) who advanced a similar explanation for the response.

The contractile tissue responsible for this expulsion of saliva appears to be the numerous myo-epithelial cells which surround the alveoli and smaller ducts of the sheep's parotid gland (Silver, 1954). The myo-epithelial character of the contractile tissue is indicated by the similarity between the responses of the sheep's parotid and those of the mammary gland of other species to the injection of oxytocin and vasopressin. There is extensive, although indirect, evidence that in the mammary gland the myo-epithelial cells surrounding the alveoli and ducts are contractile and are responsible for the ejection of milk (Richardson, 1949; Linzell, 1952). In the mammary gland, as in the sheep parotid, oxytocin readily causes an expulsion of pre-formed secretion, whereas vasopressin has relatively little action (Cross & van Dyke, 1953; Linzell, 1955). The relative potency of the drugs in this respect is the reverse of their relative pressor activity (Gaddum, 1928).

The pharmacological evidence indicates that in the parotid gland of the sheep the myo-epithelial cells are innervated by post-ganglionic adrenergic sympathetic nerve fibres that form synapses in the superior cervical ganglion. Mammary myo-epithelium, in contrast, does not appear to be innervated; sympathetic nerve stimulation and adrenaline have no direct action on the tissue, tending only to prevent the action of oxytocin, probably by causing extreme vasoconstriction (Cross, 1953; Linzell, 1955).

Babkin (1950) concluded that part of the flow of saliva that sympathetic stimulation elicits from the submaxillary gland of the dog is due to an expulsion of saliva, and attributed this to contraction of myo-epithelial tissue. The effect in this species is more difficult to examine than in the sheep since the volume expelled is relatively small and sympathetic stimulation causes some secretion of saliva as well as its expulsion.

Adrenaline may alter the flow of parotid saliva in sheep in a number of ways. The three ways apparent from the results given above are an acceleration due either to expulsion of pre-formed saliva or to an increase in the rate of reflex secretion, and a decrease due to a depression of the rate of secretion resulting from vasoconstriction induced in the gland. The effect obtained in any one experiment depended on the dose and the site of injection.

After parasympathetic denervation of the gland or the administration of atropine, the changes in the flow of parotid saliva following adrenaline injection were similar to those caused by sympathetic stimulation. In both cases there was an increase followed by a compensatory fall in the resting flow. The response to adrenaline differed from the simple response to sympathetic stimulation in that it had two peaks. The second peak occurred about 30 sec after the first and was more gradual in development. It is not known why this second peak appeared; it may have been due either to recirculation of adrenaline or to engorgement of the gland with blood during the concurrent rise in blood pressure. As with the sympathetic response, one cannot eliminate entirely the possibility that adrenaline caused an increase in the resting parotid secretion by an action on the gland itself, but such an effect, if present, was small in comparison with the volume of saliva expelled.

In preparations in which the parotid nerve was intact, the sympathomimetic action of adrenaline was usually largely masked by the onset of a secretory response. The following observations indicate that this response was due to an action of adrenaline on the afferent side of a reflex arc, and that at least some of the elements affected lay in organs supplied by the coeliac artery. (1) The response was abolished by section of the parotid nerve or by the injection of atropine; (2) the response was greatly reduced by section of the dorsal thoracic vagus nerve; (3) injections of adrenaline into the coeliac artery or into the aorta anterior to this artery evoked a response, whereas injections posterior to the coeliac artery had little or no effect; (4) the latency of the response was shortest following injection of adrenaline into the aorta or coeliac artery, longer following intravenous injection, and longest following injection into the carotid artery. It is unlikely that the reticulum contraction caused by the injection of adrenaline was responsible for excitation of the receptors, since the secretory response continued vigorously for some minutes after the reticulum had relaxed. Nevertheless, mechanical stimulation of the reticulum and various structures in the anterior part of the rumen is known to be an effective stimulus to parotid secretion (Clark & Weiss, 1952; Kay, 1958), and it is suggested that a large part of the secretory response is due to a direct action of adrenaline on receptors present in these areas.

Adrenaline has been reported to excite receptors in other species. Paintal (1954) has shown that the injection of adrenaline into the aorta of cats anaesthetized with chloralose may evoke a discharge in gastric afferent fibres. The smallest effective doses of adrenaline in cats (20–60 μg/kg) were, however, far greater than those which readily elicited reflex secretion by the sheep's parotid (2–5 μg/kg). A more closely analogous effect may be that described by Loewenstein (1956) who obtained a lowering of threshold, slower adaptation and spontaneous discharge of receptors in frog's skin following sympathetic nerve stimulation or the administration of adrenaline; mesenteric Pacinian corpuscles of cats were found to be similarly sensitized by adrenaline (Loewenstein & Altamirano-Orrego, 1956). In the sheep the restoration by the application of a stimulus to parotid secretion of the secretory response to adrenaline, after it had diminished following repeated injections, suggests that a certain degree of excitation of the receptors is necessary before adrenaline can have its effect.

In certain circumstances the injection of adrenaline 2 μg/kg may result in a series of reticulum contractions in decerebrate sheep (Titchen, 1958). Further work is necessary before a comparison can be made between this response and the action of adrenaline in sheep anaesthetized with chloralose.

When the sheep parotid was directly or reflexly excited, stimulation of the cervical sympathetic nerve or the injection of adrenaline into the carotid artery was found to annul vasodilation in the gland, and, probably as a consequence of the ischaemia, to depress the rate of secretion. Prolonged stimulation of the sympathetic was necessary to diminish the resting secretion. These results and conclusions are similar to those previously reported for the sheep by Coats *et al.* (1956), and are comparable with those found in other species (Langley, 1898).

SUMMARY

1. Changes in the rate of flow of saliva from the parotid gland of the sheep caused by stimulation of the cervical sympathetic nerve or by the injection of adrenaline have been examined in preparations anaesthetized with chloralose.

2. Stimulation of the cervical sympathetic nerve accelerated the continuous resting flow of saliva and was followed by a compensatory cessation of flow. It is suggested that this response is due to the expulsion of saliva and that no change in the rate of secretion occurs.

3. Similar changes in flow followed the injection of oxytocin but not of vasopressin.

4. The expulsion of saliva is attributed to contraction of the myo-epithelial cells of the gland, which are innervated by post-ganglionic adrenergic sympathetic nerve fibres.

5. The action of adrenaline depended on the site of injection and the experimental conditions. After section of the secretory nerve to the parotid or the injection of atropine, adrenaline caused an expulsion of saliva similar to that produced by sympathetic stimulation.

6. With the secretory nerve intact adrenaline caused copious reflex parotid secretion through an action on the afferent limb of a reflex arc, probably by direct excitation of receptors in the forestomach.

7. Both stimulation of the cervical sympathetic nerve and the injection of adrenaline into the carotid artery reduced parotid blood flow and could depress the rate of secretion.

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THE EFFECT OF GROWTH HORMONE AND INSULIN ON LIMB-BONE RUDIMENTS OF THE EMBRYONIC CHICK CULTIVATED *IN VITRO*

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In recent years it has been shown by several workers (Gaillard, 1942 (review of early work); Hardy, Biggers & Claringbold, 1953; Kahn, 1954; Lasnitzki, 1954, 1955; Fell & Mellanby, 1955; Sidman, 1956) that hormones which affect living animals when administered to them *in vivo* can also influence the differentiation and growth of organized tissue fragments *in vitro*. The study of such fragments in organ culture is a useful method by which the direct effects of a hormone, or the interaction of two or more hormones, upon a particular tissue can be investigated (Fell, 1953).

Pituitary growth hormone is known to affect the growth and body composition of animals. Its action is thought not to be a direct one, but to be dependent on the presence of insulin (Ketterer, Randle & Young, 1957) and possibly other endocrine secretions as well (Randle, 1957).

Three series of experiments are described in this paper. The first was done to see what effect, if any, growth hormone had *in vitro* on isolated long-bone rudiments of 7-day chick embryos. Chen (1954*a*) had already studied the effect of insulin on similar rudiments from slightly younger embryos, and in the second series of the present study his experiments were repeated with some minor modifications. In the third series, the combined action of insulin and growth hormone was compared with that of insulin alone, since Randle (1954) showed that growth hormone acts synergistically with insulin in enhancing the action of insulin on the uptake of glucose by the isolated rat diaphragm.

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METHODS

Tissue culture technique

The watch-glass method (Fell & Robison, 1929) as modified by Chen (1954*b*) for liquid media, was used.

The medium. In most of the experiments the medium consisted of cock serum to which very small quantities of other substances were added. The serum was prepared from non-heparinized cock plasma which had been kept at 0° C in waxed tubes for not more than a week, and usually only 24–48 hr. The plasma was converted into serum by removal of the fibrin by means of a Hemming filter (Wyllie, 1955) fitted with two 4-drachm (14 ml.) bottles. The asbestos pad generally used in such a filter was replaced by two layers of washed rayon net (as used by Shaffer (1956) for organ cultures). Enough glass beads (approximately 3 mm diam.) to form a layer about 8 mm thick were placed in the bottle adjacent to the side of the filter carrying the rayon net. The rubber washers usually supplied with these filters were replaced by silicone 'rubber' ones, and the whole apparatus was then sterilized by dry heat. About 8 ml. plasma was introduced into the bottle containing the beads, and clotted by the addition of 50 N.I.H. units thrombin dissolved in 0·6 ml. Tyrode solution. The clot was incubated for about 20 min at 37° C, then broken up with a glass rod, and returned to the incubator for a further 10–15 min to allow retraction of the clot. It was then centrifuged for 15 min at 2000 rev/min; the fibrin became entangled on the beads, while the fluid serum collected in the empty bottle.

In two experiments the culture medium consisted of 75 % serum and 25 % Parker's synthetic medium No. 1118 instead of undiluted serum. Parker's medium No. 1118 has the same composition as No. 858 (Healy, Fisher & Parker, 1955) except that in No. 1118 the concentration of each amino acid has been doubled.

The glucose level of the serum was determined and raised to 3 mg/ml. by the addition of an appropriate quantity of Tyrode solution supplemented with 5 % glucose; the glucose content of the synthetic medium was also made up to 3 mg/ml. 400 i.u. penicillin/ml. medium, and solutions of hormones (see below) or their solvents, were also added.

Thirteen drops of medium (22 drops = 1 ml.) were introduced into each watch-glass.

The hormones. Bovine pituitary growth hormone (GH) prepared in the Department of Biochemistry, University of Cambridge, by a modification of Wilhelmi's method (Wilhelmi, Fishman & Russell, 1948) was dissolved in sterile 0·9 % NaCl solution to give a concentration of 9 mg GH/ml. By means of a syringe fitted with a No. 22 gauge lumbar-puncture needle, 3 in. (76 mm) long, 0·02 ml. of this solution was added to each millilitre of medium immediately before use, to give a final concentration of 0·18 mg GH/ml. medium; a fresh solution of the hormone was prepared at the beginning of each experiment, and at each subcultivation. The same amount of 0·9 % NaCl solution was added to the medium used for the control cultures.

Crystalline insulin (supplied by Boots Pure Drug Co. Ltd) was dissolved in 0·003 N-HCl to give a stock solution of 1·6 i.u./ml.; 0·01 ml. of this solution was added to each millilitre of serum just before use, giving a concentration 0·016 i.u. insulin/ml. serum. The same quantity of 0·003 N-HCl was added to the control serum.

The explants. Hens' eggs were incubated for 7 days, and then the hind limbs of the chick embryos were removed and placed in Tyrode solution; the femur and tibia were dissected out and washed in several changes of this solution. The two rudiments from one limb were each placed in a drop of Tyrode solution on a piece of lens paper floating on a small amount of the same solution. The lens paper together with the explants was then transferred to a watch-glass containing the appropriate culture medium. The two rudiments from one limb were cultivated on the experimental medium containing the hormone(s), while those from the opposite limb of the same embryo were explanted on to the control medium which contained the solvent(s) of the hormonal solution(s).

Subcultivation and duration of the culture period. On the second, fourth and sixth days of cultivation the explants were removed from the lens paper, washed in two changes of Tyrode solution and transferred to fresh medium of the same composition as the previous one, on a new piece of paper. The cultivation period terminated on the eighth day, when most of the rudiments were weighed, but a few were fixed for histological examination.

Analysis of the effect of treatment

Length. With the aid of a camera lucida, drawings of known magnification (× 24) were made of the explants at the beginning of the experiment (after 1–2 hr incubation), before each subcultivation, and at the end of the culture period. The length of the bones in these drawings was measured with the help of a flexible ruler, and the actual length of each bone was then calculated. The lengths of the femur and tibia from one limb were usually added together and considered as one unit.

Weight. At the end of the period of cultivation the bone rudiments were removed from the lens paper, blotted on hard filter paper, and transferred to small glass capillary tubes about 1·5 cm long, sealed at one end, and previously dried at 102° C to constant weight. Four rudiments (the control or the experimental femora and tibiae from two embryos) were weighed together; this was necessary as the dry weight of the bones was so small. The explants were weighed wet immediately after insertion into the tubes, and were then dried at 102° C to constant weight.

Water content. The difference between the wet and the dry weights was considered to be the 'actual water content' of the bones, which was then expressed as a percentage of their wet weight, to give their 'percentage water content'.

Nitrogen content. The total nitrogen content of the dried bones was determined by the micro-Kjeldahl method. Each nitrogen estimation was made on four rudiments (two femora and two tibiae) which had been weighed together.

Statistical treatment of results. The significance of the mean differences in length, weight, etc. between the control and the experimental rudiments was established by calculating t $\left(\text{where } t = \dfrac{\text{difference between means}}{\text{s.e. of difference}}\right)$; P (the probability of the differences being due to chance) was obtained from tables for t (Fisher & Yates, 1953).

The difference in length or weight, etc. between paired rudiments was also expressed as a percentage difference:

$$\frac{\text{The mean difference between the control and experimental values}}{\text{The mean value of the smaller group}} \times 100$$

Histological techniques

At the end of the period of cultivation a few explants were removed from the lens paper, washed in Tyrode solution, fixed, embedded in paraffin wax, serially sectioned and stained. For general examination the tissue was fixed in 3% acetic Zenker's solution, and stained in Delafield's haematoxylin and Chromotrope 2R. Two pairs of insulin-treated rudiments were fixed for about 16 hr at 0° C in the following solution:

10% neutral formalin	2 ml.
Ethanol saturated with picric acid	18 ml.
Glacial acetic acid	0·6 ml.

The sections were subsequently stained by the periodic acid–Schiff method to demonstrate the presence of glycogen (Lillie, 1954).

RESULTS

The femora and tibiae of 7-day chick embryos consist of cartilaginous rudiments 2–3 mm long. During cultivation on serum containing 3 mg glucose/ml. they usually doubled their length in 6–8 days, the rudiments from the smaller embryos tending to do so sooner than those from the larger ones. The tibiae grew more quickly than the femora.

The effect of growth hormone

Ninety-two pairs of rudiments from 46 embryos were used in six experiments to determine the effect of growth hormone alone. In four experiments 26 pairs of femora and 26 pairs of tibiae were cultivated on cock serum, and in two experiments, in which 20 pairs of femora and 20 pairs of tibiae were grown, the undiluted serum was replaced by 75% serum and 25% synthetic medium No. 1118; 0·18 mg growth hormone (in 0·9% NaCl)/ml. was added to the two experimental media, while the paired control explants were cultivated on media containing the same volume of 0·9% NaCl. The effect of the hormone was substantially the same in both media and is shown in Tables 1 and 2.

At the end of the period of cultivation (8 days) there was no significant difference between the lengths of the treated and the untreated rudiments (i.e. the combined lengths of the femur and tibia from each limb). In both media the treated bones were, however, slightly shorter (approximately 2%) on the second day of cultivation and this figure is significant at the 5% level in the undiluted serum and at the 2% level in serum supplemented with synthetic medium.

In both media the mean wet and dry weights of the GH-treated bones were higher than those of the controls and the differences, though small, are highly significant. The treated bones contained more water, but there was no difference between their mean percentage water content and that of the controls.

The nitrogen content was estimated only on rudiments which had been cultivated on serum supplemented with synthetic medium, and the treated bones were found to contain significantly more nitrogen (13%) than the controls. There was, however, no significant difference when the nitrogen content was expressed as a percentage of their wet or dry weights. There was no effect of the hormone on the histological appearance of the rudiments.

The effect of insulin

A total of 17 pairs of femora and 17 pairs of tibiae were used in two experiments, in which the medium was undiluted serum. The results of treatment with 0·016 insulin/ml. serum are shown in Table 3 and Fig. 1.

At the beginning of the experiment there was more fibroblastic out-growth round the insulin-treated rudiments, which caused them to adhere more firmly to the lens paper than did the controls. After 4–6 days in culture the experimental explants were much broader and had larger epiphyses than the controls (Figs. 2, 3).

Insulin stimulated the increase in length of the rudiments at the beginning of the experiment, but retarded it in the second half of the culture period. Thus when the combined lengths of the femur and tibia were plotted against time (Fig. 1) the insulin-treated rudiments were seen to be 3·7% longer than the controls on the second day, but on the eighth day they were 5·5 % shorter. This graph also shows a typical growth curve for a 7-day femur and tibia cultivated in cock serum without the addition of any hormone.

The effect of insulin was much more uniform on the femora than on the tibiae. Some of the treated femora became slightly bent during cultivation, while the controls usually remained fairly straight (Fig. 2). On the other hand, many of both the control and the experimental tibiae became rather bent and distorted (Fig. 3). This was probably the result of a lack of continuity of the perichondrium surrounding the cartilaginous rudiment. When the cartilage

TABLE 1. The mean difference in length, weight, etc. between limb-bone rudiments cultivated in serum to which 0·18 mg GH/ml. was added, and their paired controls cultivated in serum without addition of the hormone

		No. of observations	Percentage difference between means	Mean difference ± S.E.	*t*	*P*
Length (mm)	Initial	26	−0·3	−0·01 ± 0·12	0·09	—
	2nd day	26	−1·8	−0·13 ± 0·06	2·12	*
	4th day	26	−0·1	−0·01 ± 0·08	0·12	—
	6th day	26	−0·9	−0·10 ± 0·16	0·68	—
	8th day	26	−0·2	−0·03 ± 0·23	0·15	—
Wet weight		11	8·5	1·186 ± 0·193 mg	5·64	***
Dry weight		11	6·5	0·088 ± 0·012 mg	7·97	***
Total water content (mg)		11	8·7	0·988 ± 0·188 mg	5·32	***
Water content as % of wet weight		11	0·2	0·21 ± 0·12 %	1·75	—

Each observation of length made on 1 femur + 1 tibia; all other measurements made on 2 femora and 2 tibiae. S.E., standard error of mean; –, $P > 0·05$ (not significant); *, $P = 0·05 - 0·01$; **, $P = 0·01 - 0·001$; ***, $P < 0·001$ (highly significant).

TABLE 2. The mean difference in length, weight, etc. between limb-bone rudiments cultivated in serum supplemented with 25 % Parker's synthetic medium to which 0·18 mg GH/ml. was added, and their paired controls cultivated in a similar medium, but without the addition of the hormone

		No. of observations	Percentage difference between means	Mean difference ± S.E.	*t*	*P*
Length (mm)	Initial	20	0·4	0·02 ± 0·04	0·50	—
	2nd day	20	−2·1	−0·17 ± 0·07	2·56	*
	4th day	20	1·9	0·20 ± 0·10	1·95	—
	6th day	20	1·25	0·13 ± 0·14	0·90	—
	8th day	20	1·4	0·17 ± 0·17	1·00	—
Wet weight		10	10·6	1·597 ± 0·440 mg	3·61	**
Dry weight		10	7·6	0·122 ± 0·036 mg	3·39	**
Total water content		10	10·8	1·458 ± 0·400 mg	3·64	**
Water content as % of wet weight		10	0·3	0·28 ± 0·16 %	1·77	—
Total nitrogen content		10	13·2	21·1 ± 5·4 μg	3·95	**
N_2 as % of wet weight		10	2·5	0·03 ± 0·04	0·72	—
N_2 as % of dry weight		10	5·7	0·57 ± 0·32	1·74	—

Length measured on 1 femur + 1 tibia; all other measurements made on 2 femora + 2 tibiae. Conventions as in Table 1.

TABLE 3. The mean difference in length, weight, etc. between limb-bone rudiments cultivated in serum to which 0·016 i.u. insulin/ml. was added, and their paired controls cultivated in serum without addition of the hormone

		No. of observations	Percentage difference between means	Mean Difference ± S.E.	*t*	*P*
Length (1 femur + 1 tibia, mm)	Initial	17	−0·3	−0·02 ± 0·05	0·48	—
	2nd day	17	3·7	0·29 ± 0·08	3·57	**
	4th day	17	0·5	0·5 ± 0·15	0·31	—
	6th day	17	−1·9	0·21 ± 0·28	0·74	—
	8th day	17	−5·5	−0·63 ± 0·26	2·41	*
Length (1 femur, mm)	Initial	17	−0·1	0·00 ± 0·03	0·00	—
	2nd day	17	6·3	0·24 ± 0·05	4·38	***
	4th day	17	2·4	0·11 ± 0·04	2·50	*
	6th day	17	−1·2	−0·06 ± 0·05	1·28	—
	8th day	17	−8·8	−0·48 ± 0·06	7·57	***
Wet weight		8	28·4	4·186 ± 0·265 mg	15·8	***
Dry weight		8	17·2	0·272 ± 0·026 mg	10·3	***
Total water content		8	29·8	3·914 ± 0·243 mg	16·1	***
Water content as % of wet weight		8	1·06	0·95 ± 0·09	10·9	***
Total nitrogen content		8	21·9	34·0 ± 8·7 μg	3·9	**
N_2 as % of wet weight		8	−5·0	−0·05 ± 0·05	1·00	—
N_2 as % of dry weight		8	4·2	0·41 ± 0·47	0·88	—

With the exception of the lengths, each observation was based on 2 femora + 2 tibiae. Each measurement of length was made on either 1 femur + 1 tibia, or 1 femur alone. Conventions as for Table 1.

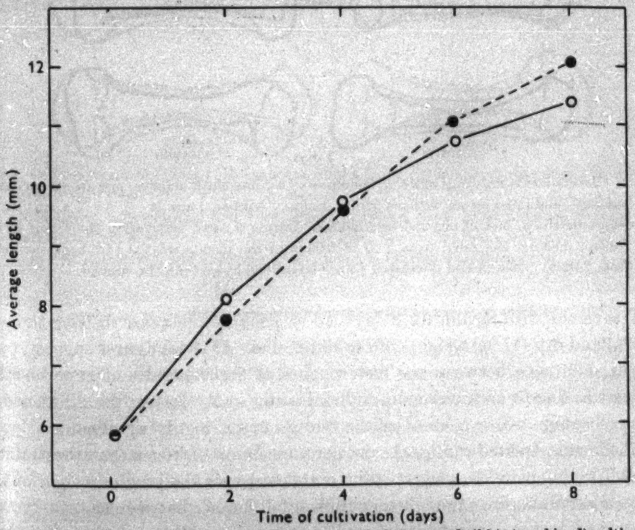

Fig. 1. The effect of insulin on the average length of the femora and tibiae (combined) cultivated *in vitro*. ●—●, control; ○—○, insulin-treated.

burst through the perichondrium growth no longer seemed to be as controlled as when the latter remained intact; such abnormal rudiments were almost always longer than their pair-mates with an intact periosteum. This abnormality tended to invalidate the tibial lengths, so the femoral lengths were analysed alone (Table 3). The insulin effect on the length was greater than when the tibiae were included; on the second day the treated bones were 6·3% longer, and on the eighth day 8·8% shorter than the controls.

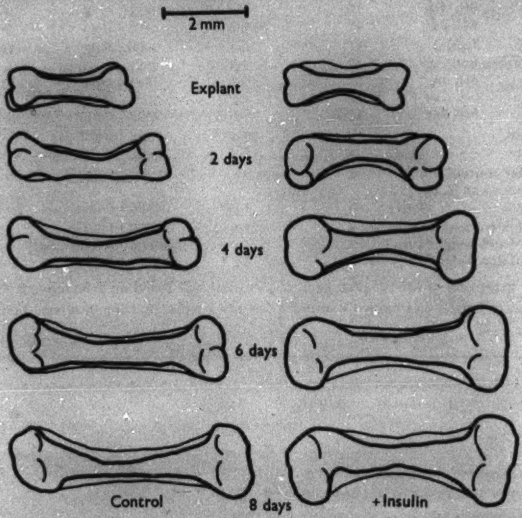

Fig. 2. Camera lucida drawings of living femora from a 7-day chick embryo, one grown in control medium (undiluted serum without addition of any hormone) and the other in a medium to which 0·016 i.u. insulin/ml. had been added. During 8 days' cultivation the femur in the control medium elongated from 3·00 to 6·08 mm; the insulin-treated rudiment elongated from 2·92 to 5·58 mm and developed rather larger epiphyses than the control.

Treatment with insulin for 8 days led to a marked increase in both the wet (28%) and dry (17%) weights of the rudiments. As these figures suggest, part of the difference between the wet weights of the control and experimental bones was due to an increased uptake of water in the latter; the difference in the percentage water content of the two groups is highly significant.

The insulin-treated rudiments also contained more nitrogen than the controls (21·9%); this increase was probably correlated with the greater weight of the treated explants, since there is no significant difference between the two groups when the nitrogen content is expressed as a percentage of the wet or dry weights.

One pair of femora and one pair of tibiae only were stained by the periodic acid-Schiff technique. This indicated that insulin treatment led to a marked deposition of glycogen in the hypertrophic cartilage, which did not occur in the controls. The cells in the hypertrophic zone of the treated rudiments were also larger than those in a comparable area of the controls. The flattened-cell zone of the experimental explants was less well defined and tended to contain less glycogen than that of the explants cultivated on the control medium.

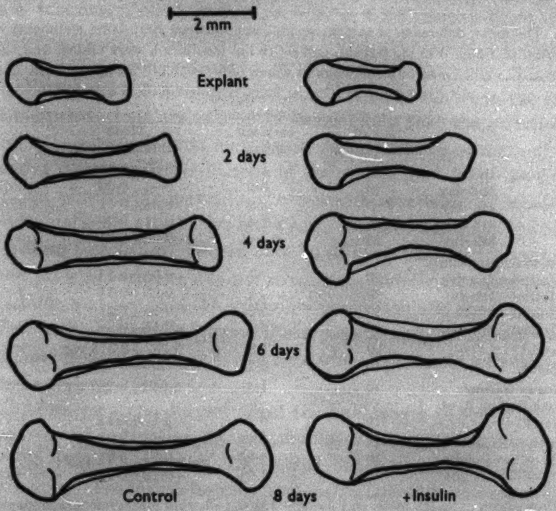

Fig. 3. Camera lucida drawings of living tibiae from a 7-day chick embryo, one grown in control medium (undiluted serum without addition of any hormone) and the other in a medium to which 0·016 i.u. insulin/ml. had been added. During 8 days' cultivation the tibia in the control medium elongated from 3·00 to 6·33 mm; the insulin-treated rudiment elongated from 2·92 to 6·00 mm and developed rather larger epiphyses than the control.

The effect of growth hormone plus insulin compared with that of insulin alone

Two experiments were done to see if growth hormone and insulin together had a greater effect on embryonic chick limb bones *in vitro* than had insulin alone. The medium for the experimental cultures consisted of undiluted serum to which were added 0·18 mg GH and 0·016 i.u. insulin/ml.; the serum for the control cultures contained the same quantity of insulin and 0·02 ml. 0·9 % NaCl (the solvent for GH)/ml. The femora and tibiae from twenty 7-day embryos were cultivated for 8 days.

During development *in vitro* both the control and the experimental explants showed a typical insulin effect, the rudiments becoming slightly bent and having large epiphyses. There was no difference in appearance or in amount of outgrowth between the two groups of cultures.

For analysis of the effect on the length of the rudiments, the femur and tibia from each limb were considered as one unit, and two femora and two tibiae were weighed together as usual. The results are shown in Table 4. It can be seen that although the mean initial length of the experimental rudiments was only 0·09 mm (1·7%) longer than that of the controls, the difference is statistically significant. It was therefore necessary to re-analyse the data to see if this difference increased or decreased significantly during treatment, and this was done by basing the analysis on the increase in length between the initial and any given succeeding day. It was shown that 0·18 mg growth hormone in the presence of 0·016 i.u. insulin had no effect on the lengths of the rudiments, except perhaps on the fourth day when the increase in length achieved by the GH + insulin-treated rudiments was 7% greater than that achieved by the paired controls treated with insulin alone.

TABLE 4. The mean difference in length, weight, etc. between limb-bone rudiments cultivated in serum to which 0·18 mg GH/ml. and 0·016 i.u. insulin/ml. were added, and their paired controls also cultivated in serum, but to which only 0·016 i.u. insulin/ml. was added

		No. of observations	Percentage difference between means	Mean difference ± S.E.	*t*	*P*
Length (mm)	Initial	20	1·7	0·09 ± 0·03	3·00	**
	2nd day	20	0·5	0·04 ± 0·06	0·67	—
	4th day	20	3·9	0·34 ± 0·11	3·09	**
	6th day	20	2·4	0·24 ± 0·20	1·19	—
	8th day	20	−0·6	−0·06 ± 0·24	0·4	—
Increase in length (mm)						
	Initial—2nd day	20	−2·1	−0·05 ± 0·13	0·37	—
	Initial—4th day	20	7·1	0·25 ± 0·11	2·21	*
	Initial—6th day	20	3·31	0·15 ± 0·21	0·75	—
	Initial—8th day	20	−2·8	−0·15 ± 0·24	0·60	—
Wet weight		10	1·3	0·237 ± 0·318 mg	0·75	—
Dry weight		10	−0·2	−0·003 ± 0·023 mg	0·12	—
Total water content		10	1·5	0·249 ± 0·297 mg	0·84	—
Water content as % of wet weight		10	0·2	0·18 ± 0·11	0·71	—
Total nitrogen content		10	−1·71	−3·0 ± 11·1 μg	0·27	—
N_2 as % of wet weight		10	−2·85	−0·03 ± 0·07	0·40	—
N_2 as % of dry weight		10	−1·24	−0·12 ± 0·73	0·18	—

Each observation of length made on 1 femur + 1 tibia; all other measurements made on 2 femora + 2 tibiae. Conventions as for Table 1.

There was no significant effect of the treatment on the weight, water or nitrogen content of the bones. No assessment of initial differences could be made, but this is unlikely to affect the general conclusions drawn, for the following reason. At the time of explantation the experimental rudiments were a little longer than the controls, and therefore, if there were any difference in initial weight or nitrogen content, it would probably have been in the same direction; at the end of the cultivation period, however, the mean wet weight of the treated rudiments was only slightly more, and the mean dry weight and nitrogen content slightly less than those of the controls.

DISCUSSION

The results just described show that growth hormone exerts a direct action on isolated limb-bone rudiments *in vitro*. Chen (1954*a*) demonstrated that insulin is easily inactivated in tissue culture (e.g. by incubation with embryo extract), and it is possible that growth hormone also may not be available to the explants unless some precautions are taken. It seems likely that the activity of the hormone in these experiments may be dependent on one or more of several experimental factors: (i) the absence of embryo extract from the medium; (ii) a method of serum preparation which avoids incubation of whole blood, with its enzymatically very active buffy coat; (iii) the addition of the hormone to the medium just before use. It may be of some importance that a fresh solution of growth hormone was prepared each time the hormone was added to the medium, and also that the solution was squirted into the serum through a long needle; P. J. Gaillard (personal communication) has since shown that parathormone is much more active *in vitro* when introduced into the medium through a fine jet.

Growth hormone alone did not affect the final length of the bones, but on the second day of cultivation, in both undiluted serum and in serum supplemented with Parker's medium, the treated bones were significantly shorter than the controls. The reason for this temporary inhibitory action is not known. Verdam (1946) found that growth hormone did not increase the growth in length of rat limb bones *in vitro*, but he reported a stimulating effect on the differentiation of the rudiments. An effect similar to the latter was not observed in the few explants of the present series which were studied histologically, nor was it seen in an extensive series of experiments on chick limb-bone rudiments *in vitro*, previously made by J. M. Chen (unpublished), who could find no direct effect of this hormone. His experiments were very similar to those described in this paper. He cultivated limb-bone rudiments from 6–7 day chick embryos on plasma and embryo extract, or on plasma clotted with thrombin, and treated the rudiments with growth hormone (0·018–0·45 mg/ml. medium). The lengths of the bones were unaffected by the hormone except in one experiment involving 10 pairs of rudiments, in which all the treated explants were longer than their paired controls after eight days in culture. Chen examined histologically all the explants that he cultivated, but he did not observe any effect on their differentiation.

A study of the effect of growth hormone and anterior pituitary extracts on the unorganized growth of tissue fragments has been made by several workers, some of whom have observed a growth-stimulating effect (Semura, 1931; Smolders, 1938, 1939, as cited by Gaillard, 1942), some no effect (Trowell & Willmer, 1939; Davidson & Waymouth, 1942, 1943), while Leslie (1952) found that the hormone had an inhibitory action.

Blumenthal and his co-workers in a series of publications (Hsieh, Wang & Blumenthal, 1952; Wang, Hsieh & Blumenthal, 1953; Blumenthal, Hsieh & Wang, 1954) showed that injection of growth hormone into eggs (5–40 μg/egg) increased the body weight and ribonucleic acid content of the whole embryo, and also raised the blood-sugar level. The effect of the hormone on the tibiae was studied in some detail; those of the treated embryos weighed more than those of the controls, and this difference was much more striking than the small differences in the lengths and widths of the bones that were also observed. The hormone caused a marked increase in the mitotic activity of the periosteum and of the proliferative zone of the cartilage, but the nitrogen content of the treated bones per unit weight was usually lower than that of the controls.

In vitro the weight of the treated rudiments was also higher than that of the controls, and so too was the total nitrogen content of the rudiments treated with growth hormone in the medium supplemented with amino acids, though there was no difference in the nitrogen content per unit weight. The nitrogen content of the rudiments cultivated on undiluted serum was not determined, as the difference in dry weight between the treated and control explants was so small. Although it is unwise to make comparisons between experiments, it may be of some significance that the difference in weight was greater in the medium supplemented with amino acids than in the undiluted serum.

Growth-hormone treatment *in vivo* tends to increase the uptake of water by the tissues, but there was no evidence of this *in vitro*: the water content per unit wet weight of the treated bones was no higher than that of the controls, although, consistent with the treated bones being heavier, their actual water content was greater. Insulin, on the other hand, significantly increased the water content per unit weight of the cultivated bones.

From these results it appears that the direct action of growth hormone *in vitro* is probably partly due to a stimulation of protein synthesis. It is now widely accepted that this hormone can exert its anabolic effect on protein only in the presence of an adequate amount of insulin (Ketterer *et al.* 1957). In the cultures, therefore, there was probably sufficient insulin in the serum or bound in the tissue of the explants to allow this effect to manifest itself. It would be interesting to repeat these experiments with an entirely insulin-free (synthetic) medium.

The increase in weight and nitrogen content of the insulin-treated rudiments cultivated *in vitro* lends support to Salter & Best's view (1953) that insulin can act as a growth hormone; they found that it stimulated rapid growth and also increased nitrogen retention (Lawrence, Salter & Best, 1954) in hypophysectomized rats. Growth-promoting effects of insulin on tissue undergoing unorganized growth in culture have been reported by Gey & Thalhimer (1924), Latta & Bucholz (1939), Haam & Cappel (1940) and by Leslie & Davidson (1951).

In the organ culture experiments reported here, the effects of insulin differed from those obtained by Chen in the following respects: the final length of the rudiments was reduced less than in Chen's cultures (1954*a*), and the treated rudiments were much less bent. In the present series of experiments the hormone stimulated growth during the early part of the cultivation period, and the double shafts observed by Chen in some of the treated explants, especially the radius, were never seen. These divergencies in the results are probably due to differences in the medium and in the size of the embryos: Chen used cock plasma and embryo extract, or plasma clotted with thrombin, and his embryos were rather smaller, 6–7 days. He noted that although the treated rudiments were shorter, their epiphyses were wider, so it seems very likely that they were also heavier, although he did not weigh them.

Landauer & Bliss (1946), Landauer (1947*a*, *b*, 1953) and Duraiswami (1950) think that the effect of insulin is very closely related to the developmental stage of the embryo. Both these authors found that injection of insulin into eggs produced micromelia in the embryo, and Landauer (1953) reported that the treated embryos weighed less than the controls. Duraiswami also observed bending of the shaft especially in the tibiae.

The histological findings, though based on very few rudiments, strongly suggest that insulin treatment promoted glycogen deposition in the hypertrophic cartilage. Glycogen is not normally present to any great extent in hypertrophic avian cartilage, either *in vivo* or *in vitro*, although there is much in hypertrophic mammalian cartilage. Sidman (1956) found that insulin treatment increased the amount of glycogen and lipid deposited in organ cultures of brown fat. In the limb-bone rudiments used in the present experiments, insulin also appeared to stimulate the hypertrophy of the cartilage cells, in contrast to Chen's observations (1954*a*) which indicated that hypertrophy and differentiation were retarded in the rather younger rudiments that he studied.

It is well known that many highly purified and even crystalline preparations of insulin are contaminated with a hyperglycaemic factor (HGF). It seems unlikely, however, that the results obtained in the present series of experiments are due to this, as Chen (1954*a*), also using Boots' crystalline insulin, proved that the effect of the hormone in his experiments was not due to contamination with HGF.

In the experiments in which the experimental serum contained added insulin and growth hormone while the control received only insulin, it seems likely that the absence of any observable effect of growth hormone may have been due to too high a dose of insulin, which itself had a big effect. Such a dose may have promoted the maximum amount of growth possible in a medium composed only of serum and glucose. Leslie (1952) obtained a similar result, however, using these two hormones on tissue cultures of embryonic chick heart fibroblasts, and Bullough (1954) found that growth hormone and insulin counteracted each other in their effect on epidermal mitotic activity *in vitro*. It would, however, be interesting to repeat the experiments on the bone rudiments with smaller doses of insulin to see if growth hormone under those conditions would enhance the effect of insulin.

SUMMARY

1. Growth hormone and insulin both affect the growth of embryonic femora and tibiae explanted from 7-day old chick embryos and cultivated *in vitro* for eight days on cock serum.
2. The wet and dry weights, and the total nitrogen content of the rudiments treated with 0·18 mg growth hormone per millilitre of medium were significantly greater than those of their paired controls, but the length of the bones was unaffected.
3. 0·016 i.u. insulin/ml. stimulated the growth in length of the bones at the beginning of the culture period, but the final lengths of the treated rudiments were shorter than those of the controls. Insulin, however, increased considerably the wet and dry weights and the nitrogen content. It also increased the water content per unit weight, and the amount of glycogen deposited in the hypertrophic cartilage.
4. 0·18 mg growth hormone plus 0·016 i.u. insulin/ml. medium had no greater effect on the limb-bone rudiments than 0·016 i.u. insulin alone.

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ION FLUXES DURING THE ACTION POTENTIAL IN *CHARA*

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Cells from the internodes of the fresh-water alga *Nitella* have been subjected to electrophysiological analysis by various investigators (Hormann, 1898; Osterhout & Harris, 1928; Blinks, 1930; Weidmann, 1949) and to a lesser extent studies of this sort have been made on the related genus *Chara* (Osterhout & Hill, 1940). Because the findings in these two forms are in many respects similar, it is convenient to treat much of the information in the literature as applicable to both. The resting membrane potential is rather greater than 100 mV (inside negative), and about equal to the propagated action potential, as measured with external electrodes. The rising phase of the spike is considerably more rapid than the falling phase. Conduction velocities are of the order of 1–2 cm/sec and the duration of the action potential is about 15 sec. The time scale of an action potential is, therefore, about a thousand-fold slower than that of a squid axon at 6·5° C.

Internodal cells of *Nitella* and *Chara* differ in that most species of this latter form possess a cortical layer of numerous small cells covering the cell wall. The wall has a thickness of 10–20 μ and covers the outer cytoplasmic membrane, while underlying this structure is a cytoplasm about 10–20 μ thick, then a vacuolar membrane and below this a large central vacuole. The material used in this investigation has been identified as *Chara globularis*, a corticated species. From a morphological point of view it should be referred to as a tissue. The experimental data to be presented show, however, that the cortical cells outside the cell wall do not act as an important barrier to ion fluxes, while the internodal cell is a physiological unit; we shall use the word 'cell' in what follows as more properly descriptive of the behaviour of the organism.

Resting values for Na^+ and K^+ transfers into *Nitella* have been measured by Brooks (1939) and by Holm-Jensen, Krogh & Wartiovaara (1944) who found that K^+ transfer was considerably more rapid than that of Na^+, in confirmation of the findings of Osterhout & Harris (1928) that K^+ was in general much more effective in depolarizing the cells than was Na^+. Our work has been concerned with the measurement of ion flows taking place during rest and during an action potential in *Chara*, and with the correlation of such changes with gradients in the electrochemical potential of the ions involved.

METHODS

Cells of corticated *Chara* were collected from ponds and stored in pond water in a refrigerator at 14° C, under continuous low level illumination from a 14 W fluorescent tube. Some days before use the strands of cells were clipped into individual inter-nodal cells and these were stored in fresh pond water. Cells used for experiments were 0·6–1·0 mm in diameter and about 50 mm long.

Analyses of pond water and cell sap for Na^+ and K^+ were made with a flame photometer, and Cl^- was estimated by titration with $Hg(NO_3)_2$ using the method of Schales & Schales (1941). Ion influx measurements were made by immersing a cell in solutions containing either ^{22}Na, ^{42}K, or ^{36}Cl for various periods of time and then washing in distilled water. The cell was then mounted on a slide designed to secure reproducible geometry and counted with a Geiger–Müller tube. At the end of the experiment, one end of the cell was cut off and 10 mm^3 of cell sap was removed for counting and analysis in order to determine its specific activity. Ion efflux measurements were made in two ways: by immersing a radioactive cell in 30 ml. of inactive experimental solution the loss of radioactivity over various periods of time could be followed by repeatedly counting the cell, while the solutions into which the radioactive ions were lost were concentrated and counted separately. At the end of an efflux experiment, the sap was collected and analysed for specific activity. When ion flux measurements were to be correlated with ion movements during the action potential, the cell was mounted in a lucite trough so arranged that its entire length except for about 3 mm at one end was immersed in the experimental solution. The recording electrodes (Ag–AgCl electrodes filled with the experimental solution) were placed in contact with the cell in the bath; the cathode of the stimulating circuit was placed on the 3 mm end of the cell projecting out from the bath, and initiated excitation at this point. The response at the recording electrodes, although much shunted by the bath, was measurable. Stimulation with 1 msec square-wave pulses was usually carried out at a rate of 1/min, although lower frequencies were sometimes used.

Radioactive ion uptake curves were also measured for cell walls with their cortication. In such cases, one end of the cell was cut off close to the node and the sap gently expressed. The wall was then immersed in distilled water for 24 hr and then transferred to a radioactive solution. Uptake and loss of radioactive ions was followed by counting the wall at various times after its immersion in either radioactive or inactive solutions.

Because of the very high longitudinal resistance of the cell wall in dilute (*ca.* 0·5 mM) salt solutions, of the order of 1·5 MΩ cm (Blinks, 1930) it seemed likely that resting potential measurements made with external electrodes between a KCl-depolarized and an untreated region were not seriously in error. A micro-electrode study of the action potential did, however, seem important in order to establish with certainty the level of maximum depolarization. Attempts to achieve penetration with electrodes of less than 1 μ tip diameter were entirely unsuccessful because of the structural rigidity of the cell wall. A reduction of the tension on the wall by the external application of hypertonic sucrose solutions did not help micro-electrode penetration but an increase in tip size of the electrodes to 10 μ did allow penetration. The cell was usually penetrated at one end, in a direction along the axis of the cell (Fig. 1). The electrodes were filled with 100 mM-KCl corresponding to the mean Cl^- concentration of the sap. It is recognized that higher concentrations of KCl are desirable from the standpoint of the elimination of junction potentials; however, the electrode tip area is so large that diffusion of KCl into the sap can be appreciable. Such electrodes were connected via a cathode follower to an oscilloscope; a cathode-ray oscilloscope was used to follow potential changes while a direct-writing oscillograph was used for recording. For certain purposes it was convenient to use another method of measuring potential. This is shown in Fig. 1 and uses a 1·5 cm gap of flowing distilled water to provide electric insulation between two chambers, one containing 100 mM-KCl and the other an experimental solution. Soft paraffin seals were used to prevent the flow of solutions into the distilled-water chamber. Tests with thread, mounted across the chambers, showed that the resistance between the two end chambers was 3–4 MΩ when they both contained 100 mM salt solution. With a *Chara* cell mounted in the chamber, the effective membrane resistance was usually of the order of 0·4 MΩ and Ag–AgCl electrodes with 10 mM-NaCl bridges were used to connect the end chambers to a differential amplifier with 10 MΩ input impedance. For stimulating cells mounted in the flowing distilled water chamber, large Ag–AgCl electrodes were connected between the experimental chamber and the tip of the cell projecting out from the paraffin seal. The electrode in the experimental chamber was usually the cathode, except for some experiments in which anode-break excitations were studied.

Cell walls were studied electrically in two ways: if both ends of a cell were cut off, a Ag–AgCl wire 0·3 mm in diameter could be threaded through the central vacuole and the two ends of such a wire fastened together, thus forming the cell wall into a loop. This assembly could be lowered into an experimental solution such that the central half of the wall was in contact with the solution.

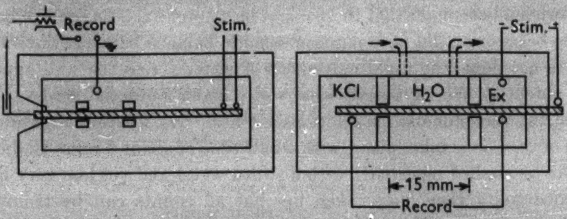

Fig. 1. On the left is shown (from above) the arrangement for recording action potentials with a micro-electrode. The cell is in a lucite trough and held between 2 V-shaped grooves. One end projects out of the bath through a paraffin seal and the micro-electrode is inserted here.

On the right is the arrangement for recording action potentials with a flowing distilled-water gap. The water flows through the centre compartment, and recording is between the KCl-depolarized region and the experimental, Ex.

When the ends of the wall had dried out somewhat, paraffin was applied to the junction between the Ag wire and the ends of the wall and measurements between the loop of cell wall and a Ag–AgCl reference electrode in the solution could be made. Because the small silver wire inside the wall showed signs of polarization with d.c. currents greater than about 100 μ, another method for electrical measurements with walls was used. Each end of the wall was cannulated by a small glass pipette and the junction sealed with soft paraffin. As before, the central half of the wall was held in experimental solutions. Ag–AgCl electrodes connected the inside of the glass pipettes and the bath solution to a source of current. The internal environment of the wall could be changed by applying gentle suction to one of the glass pipettes and thus causing the filling solution to move through the wall. Potential measurements across the wall were made with a vacuum tube voltmeter of 10^{14} Ω input impedance and potentials could be read to 1 mV. Resistance measurements were made by measuring the current flow at a series of applied potentials in the presence and in the absence of the cell wall.

RESULTS

Properties of the cell wall

Electrical measurements of the resistance of a number of cell walls equilibrated and measured in 0·1 M-KCl solution gave a value of 4 Ω cm², which, though very low when compared with membrane resistances, is appreciable for a structure about 10 μ thick, as this corresponds to a specific resistivity of

4000 Ω cm. This value is similar to that found for *Nitella* (1000 Ω cm) by Curtis & Cole (1937). Freshly prepared walls occasionally showed a rectification such that when the inside electrode was an anode the resistance was about 12 Ω cm^2, whereas it was 4 Ω cm^2 for currents in the reverse direction. A very occasional wall showed a transient to current flow, suggesting that the membrane was still capable of responding to current flow with a conductance change. Both effects disappeared if the walls were left during the night in distilled water or NaCl solution.

The wall gave no potential when the following 100 mM solutions were placed respectively on the inside and the outside: KCl/NaCl, K_2SO_4/NaCl, KCl/K_2SO_4. It was originally thought that there might be a potential between sulphate and chloride because we did not maintain osmotic balance between the solutions. If this is not done, the rapid movement of water across the wall appears to give a potential. We have never observed a stable potential across the wall.

If cell walls are immersed in solutions of ^{42}K or ^{22}Na, a rather large and rapid uptake of these ions takes place, with a half-time of about 5 min. Radioactive walls can be washed repeatedly with distilled water and yet lose very little of the radioactivity they have taken up, but all counts can be removed by immersion in inactive Na^+ or K^+ solutions. The absolute amount of material taken up is 0·5 mM/kg wet wall, and no distinction is made between Na^+ and K^+. Similar experiments with ^{36}Cl show that only a very small amount of this ion is taken up and it can all be removed with a brief wash in distilled water. Clearly the wall behaves as a cation exchange system.

The role of the cortical cells outside the cell wall in affecting the ion fluxes to be described later would appear to be small for the following reasons. The specific resistivity of the *Chara* wall with cortication is not greatly different from that for *Nitella* which has a wall without cortical cells. The wall resistance of 4 Ω cm^2 is to be compared with R_m, the membrane resistance of 200,000 Ω cm^2, as these two resistances are in series. The cortical cells might be expected to take up small amounts of radioactive ions which might confuse ion flux analyses; the evidence is, however, that owing to the large cation exchange capacity of the wall such uptake is unmeasurable. Further, the wall does not distinguish between Na^+ and K^+, whereas the intact cell does by a considerable factor. A series resistance of a magnitude similar to that of the wall occurs in the squid giant axon and is presumably due to some small impedance to ion flow brought about by the Schwann cell layer (Frankenhaeuser & Hodgkin, 1956).

Resting and action potentials

Analyses have been made of *Chara* sap and of pond water, with which the ions in the sap are presumably in a steady state. The equilibium potentials for Na^+ K^+ and Cl^- have been computed from equations (1)–(3) where brackets indicate ion concentrations, and o and i refer to outside and inside, and are shown together with the analytical values for the ions in Table 1.

$$E_{\mathrm{Na}}=\frac{RT}{nF}\ln\frac{[\mathrm{Na}^+]_o}{[\mathrm{Na}^+]_i}; \tag{1}$$

$$E_{\mathrm{K}}=\frac{RT}{nF}\ln\frac{[\mathrm{K}^+]_o}{[\mathrm{K}^+]_i}; \tag{2}$$

$$E_{\mathrm{Cl}}=\frac{RT}{nF}\ln\frac{[\mathrm{Cl}^-]_o}{[\mathrm{Cl}^-]_i}. \tag{3}$$

No comprehensive analyses of the cytoplasm for ion content have as yet been made. This is a reflexion not of the small amounts of such material available in a cell, but of the difficulty in separating it from the much greater volume of cell sap. Published analyses of the cytoplasm (Holm-Jensen *et al.* 1944; Krogh, 1946) have shown no difference in $[\mathrm{K}^+]$ when compared with sap. Such analyses as we have agree with this finding.

TABLE 1. Steady state concentrations of ions in sap

	Concentration (mM, ±S.E.)		
Ion	Pond water	Sap	Equilibrium potential of ion (mV)
K^+	0·046	65±1·9	−184
Na^+	0·15	66±1·9	−155
Cl^-	0·04	112±1·5	+202

The mean value of the resting potential of cells in pond water is −181 mV, which suggests that potassium ions in the sap are in electrochemical equilibrium with those in the environment. Sodium ion appears to be somewhat less concentrated than required by conditions of equilibrium; this cannot be attributed to a permeability difference, as we are presumably dealing with a steady-state situation. Chloride ion, in marked contrast to Na^+ and K^+, is concentrated in sap against a very large gradient in electrochemical potential.

Little is known regarding the permeabilities of the outer and inner cytoplasmic membranes in *Chara*; certain characteristics can be inferred, however, from purely osmotic considerations. The cell wall is the only structure capable of resisting the osmotic pressure under which the cells normally exist. It would appear, therefore, that cytoplasm and vacuole must be in osmotic equilibrium. Since the total osmotic pressure of the cell appears to be equivalent to 300 mM sucrose and the contribution from cytoplasmic proteins must be small, most of the osmotic pressure must be exerted by ions both in the cytoplasm and in the vacuole. One might suppose, by analogy with animal cells, that a Donnan distribution exists between the cytoplasm and the sap, with a non-diffusible anion in the cytoplasm. Such a distribution would effectively raise the cytoplasmic concentrations of Na^+ and K^+ and lower that of Cl^-. The osmotic imbalance inherent in a Donann distribution would have to be adjusted by the continuous pumping of some ion from the cytoplasm.

Attempts have been made (Walker, 1955) to demonstrate that a potential exists between the cytoplasm and vacuole in *Nitella* cells which were relatively free of chloroplasts and thus permitted visual control of the micro-electrode tip. No potential difference was observed, however. Our cells were so opaque that it was not possible to follow the micro-electrode tip visually. In several thousand punctures, however, there was never an incident of a two-step change in potential. Our conclusion is, therefore, in agreement with the measurements cited above, that the potential appears when the electrode enters the cytoplasm and is unchanged when it enters the vacuole. Some care is necessary with respect to the micro-electrode puncture of *Chara* because a 'seal' of the electrode to the cell does not develop immediately. This is suggested by the observation that whereas the membrane potential measured with the outside electrode remote from the site of insertion of the micro-electrode gives normal values, if the outside electrode is placed just at the site of insertion of the micro-electrode the measured membrane potential is often somewhat low. After some hours, a 'seal' is apparently made and the depolarized region disappears. Electrodes have been introduced that give stable potentials for 24 hr or more and, with 1 mM-NaCl solution outside, an external recording and an internal electrode give identical traces for the action potential. A further difficulty with micro-electrodes is that they tend to 'unseal' when the cell is subjected to relatively high NaCl concentrations. This has been noticed when a cell, punctured while in 1 mM-NaCl, was transferred to 100 mM-NaCl. Experimental treatments with high external osmotic pressures are better carried out with a cell mounted in a flowing distilled-water gap.

When action potentials are recorded in *Chara*, either with a micro-electrode or with a flowing-water gap, they rise from a mean resting value of −181 mV to an average value of +7 mV; the time constant of the rise is of the order of 0·1 sec, while that of the fall is about 5 sec. The rate of rise of the action potential cannot be measured properly in the flow chamber because the non-uniform excitation leads to some conduction. The electric time constant of the membrane for small voltage displacements is 0·2 sec, the cell shows sub-threshold local responses very similar to those observed for squid axon, and has a sharp threshold for excitation at a depolarization 40–60 mV below that of the resting potential.

One of the characteristics of the cell is the great variety of wave forms and amplitudes that the action potential can assume; it has been possible to find cells with resting potentials as great as −240 mV and action potentials of 200 mV. The action potential has been observed to overshoot zero by some 30 mV and various types of oscillatory behaviour of the falling phase have been noted. One experimental method of altering the wave form of the action potential measured at the cathode is simply that of stimulating with suprathreshold shocks of 1 msec duration. The essentially instantaneous displacement of the membrane potential to lower and lower absolute values serves to slow markedly the falling phase of the ensuing action potential without affecting either the rate of rise or the amplitude of the spike. The results are shown in Fig. 2.

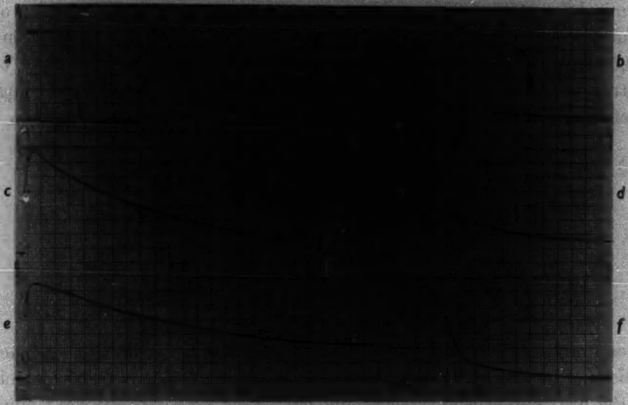

Fig. 2. Records show the response of a *Chara* cell in pond water recorded at the cathode in a flow chamber. The level of zero p.d. is shown as a horizontal bar 4 sec long; the vertical bar on the right is 50 mV. *a*, response to 1 msec stimuli of 3·3, 3·4, and 3·5 V; these yielded two local responses and an action potential. *b*, response to a stimulus of 5 V, *c*, 10 V, *d*, 20 V, *e*, 50 V and *f*, 3·5 V.

Alteration of the ionic composition of the environment. In so far as experiments over about 12 hr are concerned, bioelectric activity in *Chara* does not appear to require the presence of an ion in the external environment; the immersion of a cell for many hours in distilled water or 200 mM sucrose does not affect its ability to give an action potential. More prolonged immersion of the cell in a variety of 10 mM salt solutions does, however, lead to a reversible loss of excitability. In particular, treatment with any salt solution not containing Cl^- results in a loss of excitability; this can be restored by immersion of the cell in solutions of any chloride; choline chloride is quite satisfactory for this purpose. Table 2 shows the results obtained using twenty-four cells in each solution. Control cells in 10 mM-NaCl showed no loss of excitability after 22 days immersion. The time required for loss of excitability is much shorter than the time for half exchange of radioactive chloride with that of the sap (14 days); hence we suppose that the externally added anion has exchanged with a considerable fraction of the cytoplasmic Cl^-, thereby inducing a local depletion of this ion. The experiment indicates that Cl^- has some vital role in excitation that cannot be taken by an ion as chemically similar as I^-.

It seemed likely that the depolarization observed during the action potential in *Chara* resulted from a sudden increase in the permeability of the membrane to Cl^-. To test this hypothesis properly it would be desirable to raise the concentration of Cl^- present externally to a level where the gradient in electrochemical potential of this ion across the membrane was zero. To do this would require the external Cl^- to be 1400 times the internal concentration or 168,000 mM, whereas osmotic considerations limit the external NaCl concentration to about 150 mM. Further difficulties are encountered in going to external NaCl concentrations higher than 10–20 mM, as the membrane has a measurable permeability to cations other than K^+. At external NaCl concentrations of less than 10 mM the membrane potential appears to be controlled by the distribution of K^+ inside and out, while with 100 mM-NaCl outside there is a depolarization of about 75 mV. Such a level of depolarization does not prevent the initiation of an action potential but does make less simple the interpretation of records obtained to show that the increased $[Cl^-]_o$ decreases the level of depolarization which the action potential reaches during its peak. In this connexion it appears that 'inactivation' of the system responsible for the sudden increase of membrane chloride permeability (P_{Cl}) is not sensitive to decreases in membrane potential from resting levels of -180 to about -60 mV, because an action potential can be obtained in response to a further depolarization. Below -60 mV the membrane is largely unresponsive to stimulation.

TABLE 2. Excitability changes in chloride-free solutions

Solution (10 mM)	NaI	$NaNO_3$	Na_2SO_4	K_2SO_4
Hours of immersion needed for loss of excitability in 90% of cells	48	48	42	24
Hours of immersion needed for recovery of excitability in 10 mM/l. choline chloride, for 90% of cells	24	24	24	24

Solutions of choline chloride were found to produce less depolarization at a given concentration than did NaCl, and $MgCl_2$ appeared even more satisfactory in this respect. Cells were mounted in an insulating-gap chamber, depolarized with KCl at one end and treated with 100 mM-$MgCl_2$ at the other end. The cell was then stimulated once every 5 min and the responses recorded, and after the action potential had declined to a steady value, the cell was transferred to 150 mM-$MgSO_4$ and the action potential again recorded after a recovery period. The records from such an experiment are shown in Fig. 3. Because the resting potential declines considerably in any concentrated salt solution, the quantitative relationship between the height of the action potential and $[Cl^-]_o$ is best seen with dilute solutions, and a plot of the changes in the resting potential and in the action potential, measured as the peak level of depolarization is reached, is shown for choline chloride in Fig. 4. Increases in $[Cl^-]_o$ lead to both a diminution in the peak level of depolarization reached during an action potential, and decreases in the rate of rise of the action potential with time which, for the $MgCl_2$ records, is from 200 to 10 mV/sec. The interpretation of these curves is complicated by our lack of information regarding the diffusion delay for Cl^- that is made by the cell wall. It is also not clear to what extent the cytoplasmic $[Cl^-]$ is raised by treatment with solutions of high $[Cl^-]$. At any rate, it is usual for a high $[Cl^-]_o$ to require

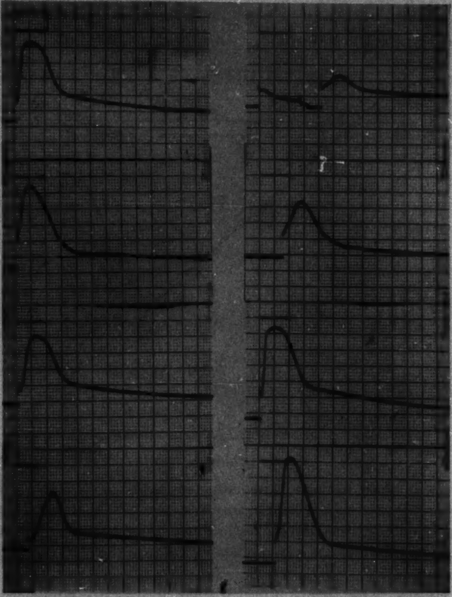

Fig. 3. The effect of 100 mM-$MgCl_2$ on the action potential. Horizontal bar shows the level of zero p.d. and is 4 sec long; vertical bar is 50 mV. The left-hand column shows, at top, the action potential of a cell in 100 mM-$MgCl_2$ after 5 min. The next three lower traces show the changes in the action potential after 10, 20 and 30 min continued immersion in $MgCl_2$. At the top of the right-hand column is the action potential of a cell after 35 min immersion in $MgCl_2$, and the three traces below show the changes in action potential after the cell had been transferred to 150 mM-$MgSO_4$ for 5, 10 and 15 min. All recording was at the cathode with the cell in a distilled-water gap chamber.

from 15 to 30 min to reduce the level of depolarization during an action potential to a steady value.

In the range of concentrations 1–10 mM none of the following ions (as chlorides) significantly affected the resting potential of *Chara*: Li^+, Na^+, Ca^{++}, Mg^{++}, or choline$^+$, whereas K^+ reduced the membrane potential an average of 55 mV. Concentrations of K^+ lower than 1 mM were very much less effective in depolarizing the membrane, a change from 0·1–1 mM gave about a 25mV decrease, while from 10–100 mM-K^+ the average change of the membrane potential was 46 mV. This figure is not highly significant, because an enhanced influx of Cl^- is to be expected and this would tend to diminish the depolarizing action of K^+. The foregoing observations would argue against there being a large Donnan potential contribution from the cell wall in series with the membrane potential, since the wall ion exchange system takes up Na^+ or K^+ without distinction but only the latter ion has any influence on the over-all potential of the cell. The membrane potential of a cell in 1–10 mM-Na_2SO_4 is the same as that for the cell in an equivalent concentration of NaCl; hence it cannot be assumed that the active uptake of Cl^- is a necessary condition for a stable resting potential, when this is measured in terms of hours.

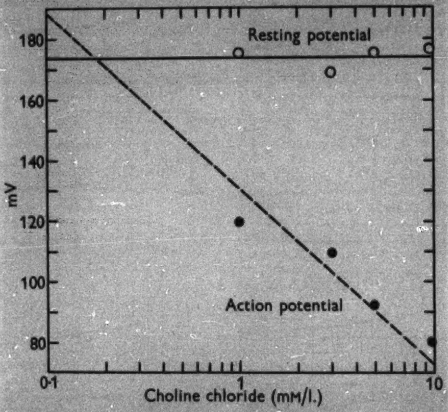

Fig. 4. The magnitude of the resting and action potentials in various concentrations of choline chloride–sucrose solutions is shown. The sucrose concentration at any point is 200 mM–2 × choline chloride concentration. The broken line has a slope of 58 mV for a tenfold change in Cl^- concentration. Recording is with a micro-electrode in the sap. Semilog. scale.

Ion fluxes at rest and during activity

Because the concentration ratios of K^+, Cl^-, and Na^+ between sap and external solution are quite different from those found in animal cells, it appears useful to calculate the flux ratios to be expected in *Chara*, on the basis that the ions move passively along their electrochemical gradients. The equation (Ussing, 1949; Teorell, 1949) for flux ratios can be written as in (4)

$$M_{out} : M_{in} = \exp(E_m - E_e)\frac{nF}{RT}, \tag{4}$$

where M is the ion flux, E_m is the membrane potential, and E_e the equilibrium potential of the ion (equations 1–3). The flux ratios for K$^+$ and Cl$^-$ for a cell in pond water are shown in Fig. 5, together with the change to be expected in Cl$^-$ flux ratios when its outside concentration is changed from that of pond water to 100 mM.

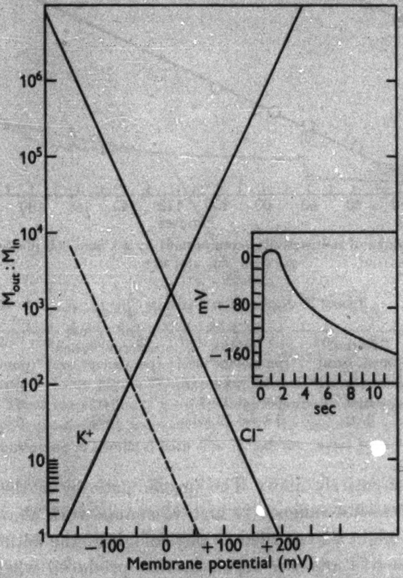

Fig. 5. The computed flux ratios for Cl$^-$ and K$^+$ are shown as a function of membrane potential for a cell in pond water. The dotted line shows the computed flux ratio for Cl$^-$ when $[\mathrm{Cl}]_o$ is 100 mM, and the sap concentration is unchanged. Semilog. scale. Inset shows a diagrammatic representation of an action potential with a resting potential of −180 mV, a threshold of 40 mV, a peak of +7 mV, and a half-time of the falling phase of 4 sec.

Measurement of the kinetics of uptake of ^{22}Na, ^{42}K, and ^{36}Cl by *Chara* gave the curves shown in Fig. 6. As the surface : volume ratio is rather small in this cell, a long time, of the order of several days, is required for equilibration of the radioactive ions with those of the sap, and uptake curves can be expected to be linear with times of the order of hours. The initial rapid uptake of K$^+$ or Na$^+$ has been shown, by other experiments, to be due to the ion exchange properties of the cell wall. Measured values for influx were, therefore, computed from the slope of the essentially linear part of the uptake curve and are shown in Table 3. As the cell wall does not have anion exchange properties, the Cl$^-$ uptake is entirely linear. The specific activity of the samples of ^{42}K available to us was not sufficiently high to enable work at concentrations of 0·04 mM, which might be regarded as physiological; the actual concentration used was 1·4 mM-KCl and the depolarization produced was of the order of 30 mV. The efflux of ^{42}K previously accumulated in the cell was followed into an external solution containing 1·4 mM-KCl, and efflux values computed from the slope of the linear part of the efflux curve (see Fig. 7). After several points on such a curve had been determined, the sap was removed from the cell for a determination of its specific activity and, with appropriate corrections for the loss of radioactivity during the experiment, the K$^+$ efflux rate could be computed. These values, together with those for ^{22}Na and ^{36}Cl, are given in Table 3.

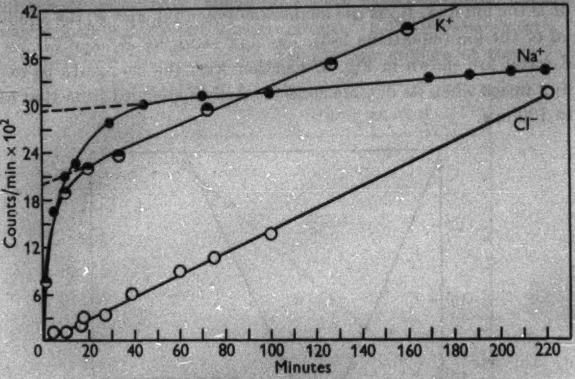

Fig. 6. The uptake of radioactivity (counts/min) by a *Chara* cell plotted against time for ^{22}Na, ^{42}K and ^{36}Cl.

TABLE 3. Resting ion fluxes in *Chara*

Ion	External concn. (mM)	Influx (pmole/cm^2.sec)	Influx/mM external concn. (pmole/cm^2.sec)	Efflux (pmole/cm^2.sec)
K$^+$	1·4	2·8 ± 0·36* (30)	2·0	6·7 ± 1·2 (66)
Na$^+$	10·0	0·7 ± 0·05 (35)	0·07	6·2 ± 1·1 (17)
Cl$^-$	35·0	1·9 ± 0·12 (21)	0·05	5·8 ± 1·0 (20)

* S.E. of mean; number of cells used is shown in parenthesis.

The values for the resting ion fluxes shown in Table 3 suggest that K$^+$ is relatively close to the steady-state condition. The K$^+$ concentration used was some thirty times that of the normal environment and the resting potential was diminished 30 mV. The observed flux ratio $M_{out}:M_{in}$ of 2·5 can be compared with that calculated from equation (4). The calculated flux ratio is about 0·1, in agreement with the finding that K^+ depolarizes the membrane relatively slightly when $[K^+]_o$ is small.

The Na^+ influx, when corrected for concentration, is about 3·5% that of K^+, while the Na^+ efflux is large and suggests, as indeed does the analysis of sap, that an active process keeps this ion from assuming a purely passive distribution in the cell. The observed $M_{out}:M_{in}$ is 9, while purely passive behaviour would demand a value of 0·006.

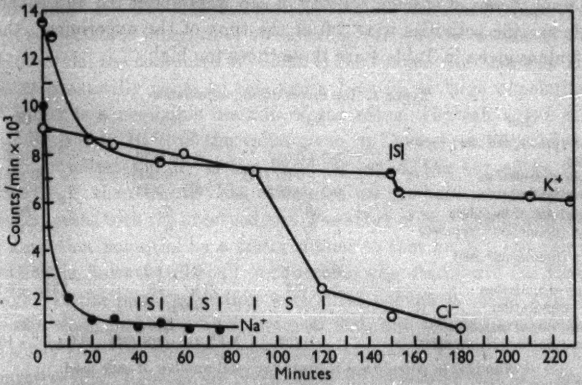

Fig. 7. The loss of radioactivity from a *Chara* cell for ^{42}K and ^{22}Na. Vertical bars denote periods of stimulation. These measurements were made by repeatedly counting the intact cell. The ^{36}Cl efflux shown represents the radioactivity lost to the immersion solution during periods of rest and stimulation.

The computed Cl^- flux ratio $M_{out}:M_{in}$ is 4000, whereas the observed value is about 3 and is presumably 1 under physiological conditions. *Chara* cells are very sensitive to mechanical stimulation and, in spite of considerable care taken in handling them, Cl^- efflux always exceeded influx. We suppose that the handling incidental to flux measurements initiated a Cl^- 'leak' in the membrane. That active transport of Cl^- is involved appears certain because it is easy to demonstrate net increases in $[Cl^-]$ in cell sap with the cell in 10 mM-NaCl, although to increase the $[Cl^-]$ of the sap will require an experiment of several days duration.

The effect of stimulation is to increase by a factor of 20–30 times the resting K^+ and Cl^- effluxes. The Na^+ influx and efflux as well as the Cl^- influx are entirely unaffected by this stimulation. The K^+ influx is reduced to an immeasurable value, but this is likely to be an artifact of the experimental arrangement. If the K^+ and Cl^- effluxes are expressed as quantity of ion leaving the cell in an impulse, the value is of the order of 10,000 pmole/cm^2.impulse. The results obtained are shown in Table 4.

In the absence of any information regarding the specific activities of K^+, Na^+, or Cl^- in the cytoplasm, we have assumed that these values were the same as sap specific activities. The validity of this assumption rests entirely on the assignment of a very low ion permeability to the outer, cytoplasmic, membrane and a very high ion permeability to the inner, vacuolar, membrane. No such assumption would be necessary if the specific activity of the sap were allowed to reach 1·0, but this requires an inconveniently long pre-loading of the cells. The actual values of specific activity of sap were about 0·3 and if the cytoplasmic specific activities were 1·0 at the time of the experiment, then the efflux values given in Table 4 are three times too high.

TABLE 4. Ion fluxes during stimulation

Ion	Na^+	K^+	Cl^-
Influx (pmole/cm^2.sec) ± S.E.			
At rest	$0·7 \pm 0·05$	$2·8 \pm 0·36$	$1·9 \pm 0·12$
During stimulation	$0·7 \pm 0·05$ (14)	0 (31)	$1·9 \pm 0·20$ (24)
After stimulation	$0·7 \pm 0·007$	$3·1 \pm 0·45$	$2·1 \pm 0·15$
Total no. of impulses	30	5	30
Influx (pmole/cm^2.impulse)	0	0	0
Efflux (pmole/cm^2.sec)			
At rest	$6·2 \pm 1·1$	$6·7 \pm 1·2$	$5·8 \pm 1·0$
During stimulation	$6·2 \pm 1·1$	$211· \pm 48$	$131. \pm 4·9$
After stimulation	—	$7·0 \pm 1·6$	—
Total no. of impulses	30	5	30
Efflux (pmole/cm^2.impulse)	0 (23)	$3·1 \pm 1·1 \times 10^4$ (26)	$0·9 \pm 0·7 \times 10^4$ (24)

Numbers in parentheses indicate the total number of cells used.

DISCUSSION

The sequence of events in the generation of an action potential in *Chara* would appear to be as follows. A depolarization of the resting membrane leads to an increase in its permeability to Cl^-: because of the steep gradient in electrochemical potential of this ion across the membrane, there is a net outward flow of negative charge which, if it exceeds the net efflux of positive ions, will depolarize the membrane further. Though E_{Cl}, which equals +202 mV, is the limit that the membrane should approach were it exclusively permeable to Cl^-, the peak depolarization is +7 mV, indicating that some other ion is contributing to the equivalent electric circuit. A decline in P_{Cl} permits the efflux of cations to return the membrane potential to its resting level.

Since Na^+ fluxes do not appear to change during bioelectric activity, attention is drawn to the movements of K^+, as they may affect the membrane potential. It is necessary to calculate whether the observed efflux of K^+ can be explained on the basis of a purely passive increase with depolarization, or whether P_K of the membrane also changes. We assume that the total membrane current I_m at the peak of the action potential (where $\mathrm{d}I_m/\mathrm{d}t=0$) is equal to $i_{\mathrm{K}}+i_{\mathrm{Cl}}=0$, where i is the ionic current, and equals $nF(M_{out}-M_{in})$. Now, the efflux of a univalent cation, on constant-field assumptions (Goldman, 1943), can be written as equation (5), where P is the permeability and C_i the inside concentration of the ion.

$$M_{out}=\frac{PC_iF}{RT}\,\frac{E_m\exp(-E_mF/RT)}{1-\exp(-E_mF/RT)} \tag{5}$$

A numerical solution of (5) for $E_m=-181$ and $+7$ mV gives 220 as the factor by which the resting efflux will be increased by the depolarization. If we take 5 pmole/cm^2. sec as the resting efflux, then at +7 mV the calculated efflux is 1100 pmole/cm^2. sec. The idealized action potential shown in the inset in Fig. 5 has been subjected to integration by summing the effluxes calculated at half-second intervals; the computed K$^+$ efflux is 3400 pmole/cm^2. impulse. The net K$^+$ loss is practically equal to this figure because at large depolarizations the K$^+$ influx is a negligible fraction of the efflux. The calculated K$^+$ loss per impulse is only 10% of the value given in Table 4, or 30% of the Cl$^-$ efflux per impulse; but if, as has been suggested, the Cl$^-$ efflux is three times too high, then the K$^+$ loss calculated on the basis of purely passive movement agrees with the observed loss. The efflux of Na$^+$ should also increase during an action potential by a factor similar to that for K$^+$, but this is not observed. It is, however, difficult to determine what fraction of the Na$^+$ efflux is passive. If the passive fraction were small enough it might still be unmeasurable when increased by a factor of 200. The conclusion that K$^+$ loss results solely from the effects of membrane depolarization on K$^+$ efflux must be a tentative one. Reasons are given below for supposing that M_{out} increases beyond values given by equation (5).

From the values of K$^+$ influx and efflux during rest and the depolarization, (30 mV), we can calculate that at -180 mV the fluxes should be equal to 3·7 pmole/cm^2. sec. Using the relationship $g_{\mathrm{K}}=F^2(M)/RT$ gives $g_{\mathrm{K}}=15$ μmho/cm^2; this may be compared with a value of 5 μmho/cm^2 from the membrane time constant in pond water. Since the Cl$^-$ fluxes are clearly not in a steady state, we should differentiate equation (5) with respect to potential in order to obtain an expression for conductance. The extra labour of using such an equation is not justified by the accuracy of the data and we may write instead that $g_{\mathrm{Cl}}=i_{\mathrm{Cl}}/(E_m-E_{\mathrm{Cl}})$. The Cl$^-$ efflux is probably never greater than 1 pmole/cm^2. sec, even though we measure a value several times this. If we use such an efflux multiplied by nF as i_{Cl}, then

$$g_{\mathrm{Cl}}=-1(96500)(1\times10^{-12})/(-0{\cdot}383)=0{\cdot}25\ \mu\text{mho/cm}^2.$$

At the peak of the action potential, where $i_{\mathrm{K}}=-i_{\mathrm{Cl}}$,

$$g_{\mathrm{Cl}}=-1(96500)(1100\times10^{-12})/(-0{\cdot}195)=545\ \mu\text{mho/cm}^2,$$

about a 2000-fold change.

The net efflux of Cl^- necessary to depolarize the membrane from -181 to $+7$ mV is 2·0 pmole/cm^2, or about 1/5000th of the observed efflux per impulse. As the shortest time for such a depolarization is about 0·1 sec, and this corresponds to a net efflux of 20 pmole/cm^2.sec, we conclude that the capacitative current is also a negligible fraction of the total ion efflux. If the efflux of K^+ is solely the result of passive movements brought about by changes in membrane potential, then the shape of the action potential describes the time course of g_{Cl}. This is unlikely to be precisely true, but it is a useful first approximation.

In interpreting the experimental data it is necessary to take into account two complications in *Chara*. The first is the presence of outer and inner cell membranes, and the second is the presence of the cell wall. We have made the assumption that we are dealing with the outer cytoplasmic membrane both because micro-electrode studies are difficult to interpret except on this basis, and because our findings of loss of excitability upon short-time immersion of cells in solutions not containing Cl^- are hard to understand if the vacuolar membrane were the one involved, since the half-time for Cl^- loss from the vacuole is about 2 weeks. That the vacuolar membrane offers some resistance to ion flow would seem to be indicated by these experiments. The loss of excitability that is observed is understandable only if the larger reservoir of Cl^- in the vacuole cannot readily enough mix with the anions of the cytoplasm to prevent it from becoming loaded with, for example, nitrate. A ready access to ions in the sap might appear necessary, as we calculate that 1 cm length of cell, with a 10 μ thick layer of cytoplasm, has 3×10^{-7} l. of cytoplasm, and if the $[K^+]$ is 50 mM then there is $1{\cdot}5 \times 10^{-8}$ mole of K, corresponding to 5×10^{-8} mole K/cm^2. This is only about 2–5 times the K^+ efflux during a single impulse, yet the cell will give a second impulse, only slightly smaller than the first, at an interval of time as short as 45 sec, and thirty impulses in as many minutes are readily obtained. As, however, the measured K^+ and Cl^- effluxes are at least 5000 times greater than those necessary to produce the observed potential change, during repetitive stimulation the cell may economize on the number of ions lost per impulse. A further reason for suspecting that it is the outer membrane that is of importance in these studies is that, for osmotic reasons, the cytoplasm must contain considerable electrolyte, and the extreme values of the measured potential across the membrane (>200 mV) would be very difficult to explain if they were developed between two regions of a cell both of which were rich in electrolyte. A second structure that complicates the analysis of both the bioelectric and ion flux measurements is the cell wall. Operationally this behaves as an unstirred aqueous layer (*ca.* 10 μ thick) and as a barrier of considerable variability to ion flow. Our present experiments have not characterized the wall sufficiently well, hence only tentative conclusions can be offered. Because the wall, when imbibed with 100 mM-KCl, has a conductance only about 2·5% that of an equivalent thickness of KCl solution, we suppose that the resistance offered to the diffusion of K^+ is similarly affected. An action potential releases of the order of 10^{-8} mole of K^+ and the diffusion of this away from the membrane surface and into the cell wall may be treated by reference to the following model. As the radius of a cell (500 μ) is very large compared with the thickness of the wall (10 μ), we can consider the membrane and the inner surface of the wall as planes in contact with each other; at zero time t, the membrane liberates Q moles/cm² of K^+ and this diffuses into the wall where we assume the diffusion constant of

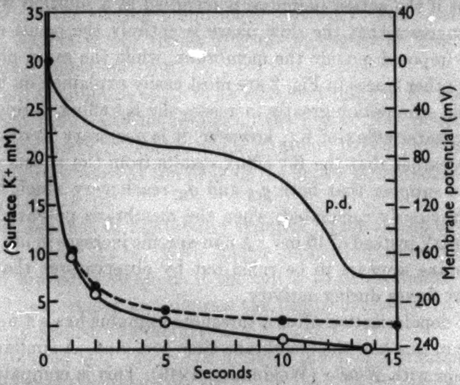

Fig. 8. The surface $[K^+]$ has been computed from the equation in the text and is shown by the dotted line. When the K^+ influx accompanying Cl^- transport is subtracted, the surface $[K^+]$ is given by the lower solid line. The potential, $E_m = \frac{RT}{F} \ln \frac{[K^+]_o}{[K^+]_i}$, given by the computed values of $[K^+]_o$, is shown by the line marked p.d.

K^+, $D = 4 \times 10^{-7}$ cm²/sec, or about 2·5% of the rate in free solution. Carslaw & Jaeger (1947) give equation (6), which relates the concentration at any distance x from the plane at x' with time, after the instantaneous release of a given amount of material.

$$C = \frac{Q}{2(Dt)^{\frac{1}{2}}} \exp\left[-(x-x')^2/4Dt\right]. \qquad (6)$$

For flow of matter in only one direction, as in the case here, the factor 2 in the denominator is to be struck out. A plot of this function for surface concentration (where the second term in the equation drops out) is shown in Fig. 8. We also assume that the cell continues to pump Cl^- inward at a rate of 2 pmole/cm².sec, that this rate is not affected by the external concentration of Cl^-, and that K^+ is likely to accompany the Cl^-. The subtraction of this influx from the amount of material in an element of wall volume (unit area and 0·1 μ thick), gives the solid curve shown in Fig. 8. The concentration of K^+ in the surface film next to the membrane can then be used to calculate the membrane potential change and such values are also shown in Fig. 8. The calculation is not likely to be very accurate at small values of time, but serves to demonstrate that it is plausible to attribute the greatly delayed repolarization time in the *Chara* action potential to the persistence of K^+ at the outer surface of the membrane.

The falling phase of a normal action potential (Fig. 2*a*) consists of a rapid and a slow phase. The slow phase disappears and the rapid phase has a shorter time constant if the action potential is recorded in a solution where $[K^+]_o$ is 5 mM. This suggests that the slow phase is entirely the result of a diffusion delay for K^+ imposed outside the membrane, while the rapid phase is partly affected. The other traces in Fig. 2 are most easily explained on the basis that suprathreshold stimulation greatly increases the K^+ efflux during an impulse. To obtain a greater efflux of K^+, however, it is necessary to discard the suggestion made earlier that the K^+ efflux results from the effects of E_m on K^+ fluxes, and to suppose that both g_{Cl} and g_K reach very much higher values than those previously calculated, when the membrane potential is suddenly displaced 100 mV instead of 40 mV. A non-specific increase in ion permeability of the membrane appears to be ruled out by observations that there is no change in Na^+ fluxes during activity.

The rise of depolarization after a threshold stimulus has a time constant of about 0·1 sec or one half the membrane time constant. A similar observation has been made with *Nitella* (Weidmann, 1949). This is compatible with the notion that the permeability of the membrane to Cl^- has increased. The usual method of testing for such a permeability change is to find that both the influx and the efflux of an ion have increased. Under the conditions obtained in our experiments, the Cl^- efflux is 6 pmole/cm^2. sec and if this is assumed to be all passive, from the steady-state flux ratio $M_{out}:M_{in}$ of $3 \times 10^{+6}$, the influx is 2×10^{-6} pmole/cm^2. sec. Even if the permeability to Cl^- were increased by a factor of 10^5, an increased influx over the influx we ascribe to transport would be difficult to measure. A further complication is the possibility of a 'long pore' effect (Hodgkin & Keynes, 1955). Even at the peak of the action potential, the computed Cl^- flux ratio is about 1500, a value too great to make measurable an increase in influx.

The apparent K^+ influx during activity drops to zero, while the efflux is enormously increased. The change in the influx may be an artifact of the method used for measurement, because unless the cell wall ion exchanger is equilibrated with radioactive solution before any measurements are made, the rapid uptake of K^+ by ion exchange obscures any uptake into the cell. Such an equilibration takes about 20 min, and at this time the cell has only a small intracellular ^{42}K specific activity. Stimulation therefore results in a large efflux of inactive K^+, and this exchanges with some of the ^{42}K on the wall; the net radioactivity of the cell decreases. The result obtained may also be a consequence of 'long pores'. Measurement of the ^{42}K efflux can be made by allowing the specific activity of cell K^+ to arise to large values, but influx measurements are necessarily inaccurate in the presence of large K^+ effluxes.

SUMMARY

1. Sodium, potassium, and chloride ions are concentrated in the sap of *Chara* so that the equilibrium potentials for these ions are −155, −184, and +202 mV, while the resting potential is −181 mV. The resting membrane resistance of 200,000 Ω cm² agrees with the measured K^+ flux in the steady state of about 3 pmole/cm². sec.

2. Threshold stimulation leads to an increased efflux of K^+ and Cl^- with no change in their influx and no change in the flux of Na^+ in either direction. The efflux of K^+ and Cl^- reach a value of about 10,000 pmole/cm². impulse. The membrane resistance at the peak of the action potential, computed from Cl^- efflux, is 2,000 Ω cm².

3. The maintenance of excitability requires no ion in the bathing solution for periods of about 12 hr. With longer immersion times Cl^- is required externally in low concentrations if the cell is to be excitable. High concentrations of Cl^- externally first depress the height of the action potential and may ultimately abolish excitability.

4. It is supposed that bioelectric activity involves a transient increase in the permeability of the membrane to Cl^- and that the depolarization produced by Cl^- efflux is followed by an increased K^+ efflux which returns the membrane potential to resting levels.

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THE RELATIONSHIP OF HISTAMINE AND 5-HYDROXYTRYPTAMINE TO ANAPHYLACTIC SHOCK IN DIFFERENT SPECIES

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Histamine is released during anaphylactic shock and accounts for many of the observed symptoms. In recent years, interest has been focused on 5-hydroxytryptamine (5-HT) as another amine involved in the anaphylactic reaction. The present study was undertaken to assess the relative importance of histamine and 5-HT in anaphylactic shock in different species. The rat is particularly suitable for this work as its tissues can be preferentially depleted of either of these amines (Parratt & West, 1957*b*) and severe shock can be consistently produced by using *Haemophilus pertussis* vaccine with the sensitizing dose of antigen (Sanyal & West, 1958). In this species the effect on the anaphylactic reaction of depletion of tissue histamine and 5-HT and of specific antagonists of these substances was therefore determined. In addition, the amine release from tissues was studied in the rat as well as in the guinea-pig, rabbit and dog.

METHODS

Female albino rats (100–150 g), adult guinea-pigs, adult rabbits and young dogs were used.

Sensitization. Fresh egg white, which had been mixed with an equal volume of normal saline (aqueous NaCl 0·9 % (w/v)) and strained, or horse serum was used as antigen. Rats were sensitized by an intraperitoneal injection of either 1 ml. of egg white or a mixture of 1 ml. of horse serum and 1 ml. of *H. pertussis* vaccine, phase I, containing $20,000 \times 10^6$ organisms (Sanyal & West, 1958). Guinea-pigs were sensitized by an intraperitoneal injection of 0·5 ml. of horse serum. Rabbits received 1 ml. of horse serum intraperitoneally on each of 6 consecutive days. Dogs were sensitized by a subcutaneous injection of 2 ml. of horse serum, followed 2 days later by an intravenous injection of 3 ml. of horse serum.

Challenge. All animals were challenged by an intravenous dose of 1 ml. of antigen. This was given 12–18 days after the last sensitizing dose in rats, 21–28 days in guinea-pigs, 14–20 days in rabbits, and 20–30 days in dogs.

Assessment of shock. The symptoms of shock in the rat were recorded as previously described (Sanyal & West, 1958). Development of asphyxia was taken to indicate the presence of anaphylactic shock in the guinea-pig. A marked fall in blood pressure indicated shock in the rabbit and dog.

Depletion of tissue amines in the rat. To deplete the skin of its histamine before challenge with the antigen, five doses of polymyxin B were given intraperitoneally over 3 days (Parratt & West, 1957*b*), commencing 9 days after sensitization. To deplete the skin and intestine of their 5-HT, one dose of reserpine (10 mg/kg) was given intraperitoneally 24 hr before the challenge. To deplete the stomach and jejunum of their histamine, a dose of 900 r total body irradiation (Eisen, Ellis & Wilson, 1956) was given 6 days after sensitization. To lower the skin histamine of several of the irradiated rats, repeated doses of polymyxin B were given during the 3 days before the challenge.

Use of specific antagonists in the rat. Thirty minutes before the challenge, rats received intraperitoneal injections of either mepyramine, a specific antagonist of histamine, or 2-bromo-lysergic acid diethylamide (BOL 148), a specific antagonist of 5-HT.

Amine release by antigen from the whole animal. Amine release by antigen was studied before and after anaphylactic shock. On the day of challenge with antigen the animals were anaesthetized with urethane (1·5 gm/kg intraperitoneally) and blood pressure recordings were taken from the carotid artery. Artificial respiration was necessary for guinea-pigs and rabbits, in order to remove a piece of right lung for extraction and assay. In dogs pieces of liver and of spleen were similarly removed for assay. Twenty to thirty minutes after the challenging dose of antigen had been given, similar pieces of tissue were removed from each animal for assay. The tissues were extracted either with trichloroacetic acid and assayed for histamine on the isolated guinea-pig ileum or with acetone and assayed for 5-HT on the isolated rat uterus, as described in detail by Parratt & West (1957*a*). All values for histamine and 5-HT in this paper refer to the bases.

Amine release by antigen from isolated tissues. This was carried out by a modification of the method of Campbell & Nicoll (1940). Pieces of tissue of similar weight from non-sensitized or sensitized rats, guinea-pigs or dogs were suspended in the organ bath (vol. 20 ml.) in which was set up an assay preparation of either guinea-pig ileum, rat uterus or rat colon. The bath fluid contained atropine (10^{-7}). The specific antigen (0·2 ml.) was then added and the contraction produced was compared with those resulting from the addition of doses either of histamine on the guinea-pig ileum or of 5-HT on the rat preparations. The experiments were then repeated with other pieces of tissue on the guinea-pig ileum in the presence of mepyramine (10^{-7}) or on the rat preparations in the presence of BOL 148 (10^{-7}).

Histological examination. Fresh tissue spreads of rat subcutaneous connective tissue and of guinea-pig phrenic nerve with its attached pleura were made before and after challenge with the antigen. They were fixed in alcohol, stained with toluidine blue and mounted (Parratt & West, 1957*a*).

RESULTS

Anaphylaxis in the rat. The results were the same whether horse serum or egg white was used as the antigen. Depletion of tissue histamine by treatment with polymyxin B only slightly lowered the average shock value of rats undergoing anaphylaxis and deaths were recorded in each group. Depletion of tissue 5-HT by treatment with reserpine likewise had little or no effect on the shock values. In sensitized rats receiving X-irradiation there was an indication that the shock produced was more intense than in control sensitized rats given the challenge, as fatal reactions were obtained much earlier. X-irradiation did not alter the shock value of rats whose histamine had been depleted by treatment with polymyxin B. The results obtained with horse serum are summarized in Table 1.

Pre-treatment with mepyramine (10 mg/kg) or with BOL 148 (4 mg/kg) or with both antagonists did not significantly reduce the severity of anaphylactic shock. The results using horse serum as antigen are shown in Table 2 and similar values were obtained for egg white.

When the amine release from isolated rat intestine by antigen was examined, it was found that both horse serum and egg white were effective in releasing substances which stimulated the guinea-pig ileum, rat uterus and rat colon, and there was no difference whether the intestine was from sensitized or non-sensitized rats.

Table 1. The effect of different treatments on the average shock value of rats undergoing anaphylactic shock. Sensitization with horse serum and *H. pertussis* vaccine; challenge with horse serum 12 days later

Treatment	Number of rats used	Average shock value (%)
None	18	100
Polymyxin B	12	81
Reserpine	9	90
X-irradiation	5	100
X-irradiation + polymyxin B	5	80

Table 2. The effect of specific antagonists of histamine and 5-hydroxytryptamine on the average shock value of rats undergoing anaphylactic shock. Sensitization and challenge as described in Table 1

Antagonist	Number of rats used	Average shock value (%)
None	18	100
Mepyramine	6	92
BOL 148	6	92
Mepyramine + BOL 148	4	100

The appearance of the mast cells of the subcutaneous connective tissue was not altered by anaphylactic shock, and even when the mast cells had been completely disintegrated by repeated injections of polymyxin B severe and often fatal shock was produced.

Anaphylaxis in the guinea-pig. Histamine is known to be released from guinea-pig lungs during anaphylaxis. In the present experiments, horse serum reduced the histamine content of the lungs of four non-sensitized animals by about 16% (range 14–25%) whereas in eight sensitized animals the average reduction was 62% (range 31–88%). The histamine release from isolated guinea-pig lungs during anaphylaxis was not studied because this fact is well established. Anaphylactic shock also degranulated and distorted the pleural mast cells, but did not alter the 5-HT content of the lungs.

In contrast to the rat experiments, the isolated rat uterus and colon were not stimulated when pieces of lungs of non-sensitized guinea-pigs were suspended in the organ bath and the antigen was added. A smooth-muscle-stimulating substance was released, however, when a piece of lung of a sensitized animal was present, and estimates of its activity, calculated as 5-HT, gave similar values on both rat preparations (0·08 μg/g tissue). The stimulant action was not observed in the presence of BOL 148 (10^{-7}), and this result further suggested that the stimulation was due to the release of 5-HT from the tissues. Release of a similar smooth-muscle-stimulating substance was also obtained from pieces of spleen of sensitized animals on the addition of the antigen but no such release occurred from aorta or oesophagus.

Anaphylaxis in the rabbit. Intravenous doses of horse serum produced no alteration in the histamine or 5-HT contents of the lungs of non-sensitized rabbits but considerably raised the levels of both amines in the lungs of sensitized animals (see Table 3).

TABLE 3. The influence of anaphylactic shock on the histamine and 5-hydroxytryptamine contents (μg/g) of rabbit lung

	Histamine			5-HT		
Rabbit no.	Pre-shock value	Post-shock value	Change (%)	Pre-shock value	Post-shock value	Change (%)
1	4·2	8·0	+ 90	1·7	4·3	+153
2	7·5	16·4	+118	2·1	6·8	+228
3	9·9	25·6	+158	7·1	20·3	+185
4	3·6	11·3	+213	1·5	2·5	+ 66

TABLE 4. The influence of anaphylactic shock on the histamine and 5-hydroxytryptamine contents (μg/g) of dog liver

	Histamine			5-HT		
Dog no.	Pre-shock value	Post-shock value	Change (%)	Pre-shock value	Post-shock value	Change (%)
1	6·8	4·4	−35	1·55	1·60	+ 3
2	14·0	2·0	−86	0·50	0·45	−10
3	13·0	5·0	−62	0·35	0·35	0
4	16·0	2·0	−82	0·45	0·45	0

Anaphylaxis in the dog. The injection of horse serum into control dogs did not produce a fall in arterial blood pressure nor did it alter the histamine or 5-HT contents of the liver. In sensitized dogs, however, the injection produced an abrupt fall in blood pressure accompanied by a considerable loss of histamine, but not of 5-HT, from the liver (Table 4). The loss of histamine was not confined to the liver, as the spleen also showed a loss of histamine, though not of 5-HT.

The isolated rat uterus was not stimulated when a piece of liver of a sensitized dog was suspended in the bath. There was also no stimulation when horse serum was added as well. Nevertheless, the dogs were sensitized since the intravenous injection of horse serum produced an abrupt lethal fall in arterial blood pressure.

DISCUSSION

The present results show that neither histamine nor 5-HT plays a role in the anaphylactic shock of the rat. This conclusion is based on the finding that the shock is little altered when the skin and intestinal histamine and 5-HT are depleted and when the animals have been pre-treated with specific antagonists of both amines. Further, the release of histamine and 5-HT from sensitized tissues on addition of antigen is no more than that from non-sensitized tissues. Tissue mast cells which contain histamine and some of the 5-HT also fail to exhibit major changes following anaphylaxis, and their complete disruption does not reduce the severity of the reaction. This conclusion is in agreement with our earlier findings (Sanyal & West, 1958). It is thus possible that anaphylaxis in the rat is mediated by substances which have not so far been identified or that the antigen-antibody reaction directly damages the susceptible cells.

A different conclusion concerning the role of histamine in rat anaphylaxis has been reached by Mota (1957). He based his conclusions on the following three points: (1) Tissue mast cells which contain histamine are disrupted during anaphylaxis. (2) A rise in plasma histamine occurs in the anaphylactic reaction. And (3), rats can be protected against anaphylactic shock by pretreatment either with chlorprophenpyridamine maleate (Alergon), an antihistamine drug, or with compound 48/80, a potent histamine liberator. Examination of his published data, however, reveals that only a proportion of the tissue mast cells showed granule extrusion with little disruption; however, such changes are frequently encountered in tissue spreads from control rats. Moreover, as shown in the present experiments, complete degranulation and disruption of mast cells as, for example, by treatment with polymyxin B, is followed by severe and often fatal anaphylactic shock. Mota did not report the plasma histamine levels of non-sensitized rats receiving the antigen (horse serum) and it is difficult to assess whether the slightly raised values of plasma histamine in some of his rats are related to anaphylaxis, as horse serum releases histamine from tissues of non-sensitized animals (Sanyal & West, 1958). Further, the protective effect of compound 48/80 (as reported by Mota) loses much of its significance as the method of sensitization used produced mild, and at no time fatal, anaphylactic shock.

Histamine release from the lungs of guinea-pigs during anaphylaxis has been confirmed (for references, see Rocha e Silva, 1955). Although horse serum releases histamine from the lungs of control guinea-pigs, the release from the lungs of sensitized animals is considerably greater. Moreover, pleural mast cells show changes during anaphylaxis which are suggestive of histamine release. There is a release of a minute amount of a smooth-muscle-stimulating substance other than histamine in the tests of anaphylaxis *in vitro* and its action is similar to that of 5-HT. As the injection or inhalation either of histamine or of 5-HT produces in guinea-pigs symptoms which resemble those found in anaphylactic shock, it is apparent that both substances may play a part in anaphylaxis in this species. Histamine is likely to be the major component responsible for asphyxial death but 5-HT may play an accessory role. The complete obstruction of the respiratory passages, which is a characteristic feature of anaphylactic shock in the guinea-pig, results from the special arrangement of the smooth-muscle fibres in the trachea and bronchi which, in the guinea-pig, in contrast to the arrangement in other species, are attached to the inner aspect of the cartilage. Both histamine and 5-HT stimulate these smooth muscle fibres *in vitro* (Brocklehurst, 1958) and their contractions in anaphylaxis may produce dove-tailing and folding of the bronchial mucosa, as reported by Schultz & Jordan (1911), resulting in respiratory obstruction and death.

Anaphylaxis in the rabbit results in a decrease in blood histamine, blood 5-HT and the number of circulating platelets (Waalkes, Weissbach, Bozicevich & Udenfriend, 1957). As rabbit platelets are rich in both histamine and 5-HT and release these two amines during the antigen-antibody reaction (Humphrey & Jaques, 1954), the increase in the histamine and 5-HT contents of the lung noted in the present experiments after anaphylaxis may be accounted for by the trapping of platelets in the pulmonary bed, as suggested by Schachter (1953).

In the dog histamine release from the liver during anaphylactic shock has been confirmed. No evidence however has been obtained to suggest that 5-HT is released either from the liver or from the spleen, although appreciable quantities of 5-HT are normally located in these two tissues in this species.

The present results show that there is considerable species variation in the relative importance of histamine and 5-HT in the production of anaphylactic shock. Both amines are involved in the rabbit but only histamine in the dog. In the guinea-pig 5-HT is involved but it probably plays a minor role. In the rat, however, neither histamine nor 5-HT appear to participate. The characteristic lesions of anaphylaxis in the rat consist of haemorrhage in the small intestine, and similar lesions have also been found in the dog and guinea-pig, especially when death is delayed as after the intraperitoneal or subcutaneous challenge with antigen (Williamson, 1936) or after an antihistamine drug (Green, 1953). Such changes therefore represent a pattern of lesions in anaphylaxis common to several species.

SUMMARY

1. The role of histamine and 5-hydroxytryptamine in the production of anaphylactic shock has been examined in rats, guinea-pigs, rabbits and dogs.

2. In rats no evidence could be found that either amine participates in the symptomatology of anaphylactic shock. Shock occurs in animals whose skin and intestinal histamine and 5-hydroxytryptamine have been depleted. Specific antagonists of both amines similarly fail to modify the severity of the shock.

3. In guinea-pigs and rabbits both histamine and 5-hydroxytryptamine are involved in anaphylactic shock.

4. In dogs the release of histamine, but not of 5-hydroxytryptamine, occurs as a result of the anaphylactic reaction.

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VASODILATATION IN THE TONGUE AND ITS RELATIONSHIP TO PLASMA KININ FORMATION

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The blood flow through the tongue, like that through the submandibular gland, is greatly increased on stimulation of the chordolingual nerve, the response being produced by parasympathetic efferent fibres of the chorda tympani (Heidenhain, 1883; Machol & Schilf, 1928; Erici & Uvnäs, 1952). The vasodilatation in the salivary gland has recently been shown to be mainly due to formation in the tissue spaces of a plasma kinin similar to bradykinin as a result of activation of the gland by the secretomotor fibres and of release of a plasma kinin-forming enzyme from the gland cells (Hilton & Lewis, 1955*a*, *b*). The present experiments show that this enzyme is also released from the tongue on chordolingual stimulation.

METHODS

The experiments were performed on cats under pentobarbitone (40 mg/kg) anaesthesia.

Experiments on the tongue. All branches of both external jugular veins were tied, except the submental veins. These were cannulated with polythene tubing, joined by a Y-piece to form a common return, for registration of venous outflow or collection of perfusate. When measuring venous outflow, the blood was passed through a photo-electric drop-counting chamber, registered by the Gaddum drop-timer and then returned into the right jugular vein. Blood pressure was registered from a cannula in a femoral artery. For perfusion experiments the carotid artery on the right side of the neck was prepared by ligation of all branches apart from the lingual, and the main trunk of the carotid was cannulated distal to the origin of the lingual branch so that the lingual artery could be perfused. When perfusion was started, a clamp was placed on the trunk central to the lingual branch. The left lingual artery was tied. Special precautions were taken to ensure that the perfusate was free from blood, because it was not possible to isolate the venous outflow from the tongue even if ligatures were tied round all the soft tissues of the neck below the tongue. In some experiments the animal's blood pressure was reduced by bleeding to 30–40 mm Hg, but as the blood pressure would sometimes rise again quite sharply blood would reappear in the perfusate. In most experiments, therefore, the animal was killed when perfusion was started.

Both lingual and both hypoglossal nerves were cut and the distal ends fixed in fluid electrodes for stimulation. The lingual nerve was stimulated by square waves of 0·5 msec duration at 10/sec, and the hypoglossal nerve by square waves of 0·1 msec duration at 40/sec.

In two experiments, the chorda tympani was divided before it joined the lingual nerve 7–10 days before the actual experiment. These operations were performed aseptically under pentobarbitone anaesthesia. In some experiments the gastrocnemius muscle and the skin of the thigh were perfused with oxygenated Locke's solution.

Perfusion of gastrocnemius muscle. The venous outflow from the muscle was isolated as described by Verzar (1912). To avoid contamination by blood the gastrocnemius was separated from the deep muscles of the lower leg and ligatures were placed round both leads of the muscle and its tendon. The femoral vein was cannulated for registration of venous outflow or collection of perfusate. A cannula was tied into the popliteal artery below the origin of the gastrocnemius branches, the artery being clamped above these branches during perfusion. The muscle was tetanized by stimulation of the peripheral end of the cut tibial nerve with square waves of 0·1 msec duration at 40/sec and supramaximal voltage.

Perfusion of skin of the thigh. These experiments were carried out on cats which, 20 days previously, had had their lumbar sympathetic chain removed aseptically under pentobarbitone. The femoral vein was tied below the origin of the saphenous branch and cannulated above it for registration of venous outflow or collection of perfusate from the saphenous vein. Adjacent branches of the femoral vein were tied. The femoral artery was cannulated below the origin of its saphenous branch, the artery being clamped above it during perfusion. The skin of the thigh was separated from the underlying tissues. The peripheral end of the cut saphenous nerve was stimulated with square waves of 1 msec duration at 20/sec and supramaximal voltage for the vasodilator effect. In all experiments the trachea was cannulated, and the cat was given heparin (10 mg/kg) before cannulating the vein for the venous outflow.

Assay of perfusate for plasma kinin-forming activity. The samples of perfusate were kept on ice until assayed. They were then incubated with pseudoglobulin solution for 1 min at 30° C and assayed against standard bradykinin (kindly supplied by Dr W. Beraldo) on the isolated rat uterus, as previously described (Hilton & Lewis, 1956).

RESULTS

Experiments on the venous outflow from the tongue

Stimulation of the chordolingual nerve is known to cause a pronounced vasodilatation in the tongue. The effect on the venous outflow of 10 sec nerve stimulation is shown in Fig. 1*a*. The vasodilator effect is only slightly reduced by atropine and thus is as atropine-resistant as the vasodilatation produced by chorda stimulation in the submandibular salivary gland.

The ganglion-blocking agent, hexamethonium bromide, almost abolishes the vasodilatation. The residual vasodilator response to chorda stimulation, after 1 mg of hexamethonium bromide given by close arterial injection, is shown in experiment Fig. 1*b*. This finding indicates that the post-ganglionic parasympathetic fibres mediating the response originate in the substance of the tongue itself.

The failure to abolish the response completely with hexamethonium is consistent with the conclusion of Erici & Uvnäs (1952) that antidromic stimulation of sensory fibres in the lingual nerve of the cat can produce a small vasodilator effect in the tongue. To examine this possibility experiments were performed on two cats 7–10 days after the chorda tympani had been divided. In these experiments lingual nerve stimulation still produced a small vasodilatation which, however, was resistant to hexamethonium.

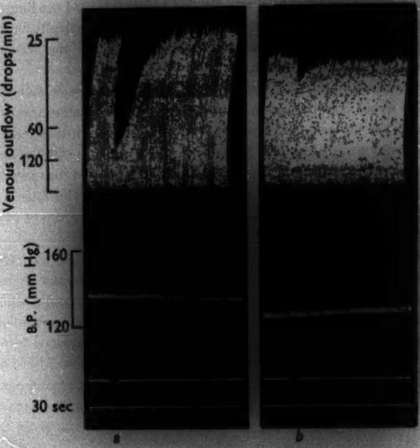

Fig. 1. Venous outflow from the tongue (upper tracing, semi-log scale) and arterial blood pressure (lower tracing) of an anaesthetized cat. The chordolingual nerve was stimulated for 10 sec before (*a*) and after (*b*) an arterial injection of 1 mg of hexamethonium bromide.

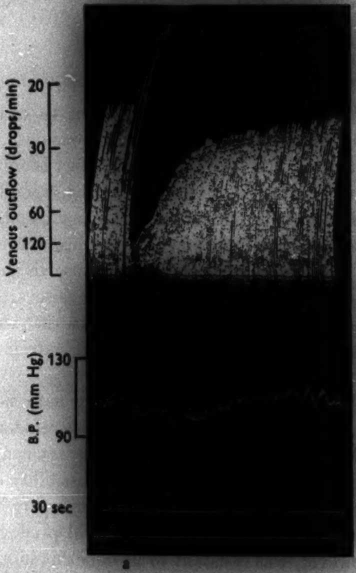

Fig. 2. Venous outflow from the tongue (upper tracing, semi-log scale) and arterial blood pressure (lower tracing) of an anaesthetized cat. The hypoglossal nerve was stimulated for 10 sec at (*a*).

Stimulation of the hypoglossal nerve. Hypoglossal nerve stimulation which produces tetanic contraction of the tongue is followed by pronounced vasodilatation. The effect of 10 sec stimulation of the hypoglossal nerve is illustrated in Fig. 2. The vasodilatation is larger than that resulting from supramaximal stimulation of the chordolingual nerve for the same period of time, and, like the functional hyperaemia in skeletal muscle of the limbs, it is not affected by either atropine or hexamethonium.

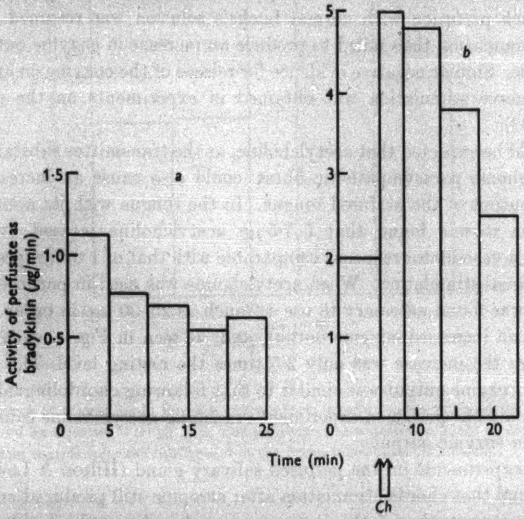

Fig. 3. Output of plasma kinin-forming enzyme from the tongue of two cats, perfused for 25 min. (*a*) Resting tongue; (*b*) tongue activated by 1 min chordolingual stimulation at *Ch*. In each experiment the perfusate was collected in 5 min periods, except for the 3 min sample collected during and in the 2 min following chordolingual stimulation.

Perfusion experiments

Tongue. When the tongue was perfused with oxygenated Locke's solution, small amounts of plasma kinin-forming enzyme were found to escape into the venous effluent at rest. The resting values expressed in μg bradykinin formed per minute of perfusion were between 0·4 and 1·0 in seven experiments, between 1 and 2 in five, between 3 and 7 in four experiments and 33 in one experiment. When perfusion was maintained for as long as 25 min, the values remained steady at this low level, as shown in Fig. 3*a*.

Following stimulation of the chordolingual nerve for 1 min, the output of enzyme from the tongue increased to 2·5–5·5 times the resting level Fig. 3*b*.

shows the histograms from an experiment in which there was a 2·5-fold increase in perfusate collected during the 1 min of chordolingual stimulation and the 2 min following. In the subsequent samples, collected over periods of 5 min each, the enzyme activity gradually returned to the original level.

This increased enzyme output, like the vasodilatation seen with normal blood circulation, results from stimulation of the parasympathetic efferent fibres of the chorda tympani. This was shown by perfusing the tongue for 5 min with Locke's solution containing hexamethonium bromide (200 μg/ml.), after which perfusion with normal Locke's solution was resumed. Chordolingual stimulation then failed to produce an increase in enzyme output from the tongue. Similar negative evidence for release of the enzyme on antidromic sensory nerve stimulation was obtained in experiments on the cat's skin (see p. 538).

It would be expected that acetylcholine, as the transmitter substance of the post-ganglionic parasympathetic fibres, could also cause an increase in the enzyme output of the perfused tongue. In the tongue with its normal blood circulation it was found that 5–7·5 μg acetylcholine infused over 1 min produced a vasodilator response comparable with that of 1 min supramaximal chordolingual stimulation. When acetylcholine was used in perfusion experiments it was found necessary to use as much as 25–50 μg, in order to obtain regularly an increased enzyme output, and, as seen in Fig. 4, even with the 50 μg dose the increase was only 2·5 times the resting level. The profile of increased enzyme output was similar to that following chordolingual stimulation. In only one perfusion experiment did 7·5 μg acetylcholine cause a small increase in enzyme output.

In the experiments on the perfused salivary gland (Hilton & Lewis, 1956) it was found that chorda stimulation after atropine still produced an increase in enzyme output, though this increase was reduced to a third or less of that found without atropine. However, when the tongue was perfused after an intravenous injection into the whole animal of atropine 0·1 mg/kg, the enzyme activity after chorda stimulation was not significantly different from the resting level.

In view of the pronounced vasodilatation produced by hypoglossal nerve stimulation in the tongue with normal blood circulation, the effect of such stimulation on the enzyme output of the perfused tongue was investigated. It was found that hypoglossal nerve stimulation for 1 min caused no increase in the output of enzyme from the perfused tongue. Histograms from a typical experiment are shown in Fig. 5.

Gastrocnemius. Similar results were obtained in experiments on the perfused gastrocnemius muscle. Small amounts of plasma kinin-forming enzyme appeared in the perfusate from the resting muscle, and no increase in enzyme

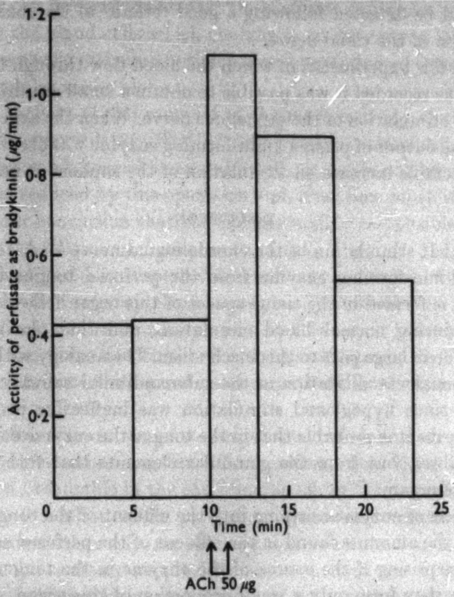

Fig. 4. Output of plasma kinin-forming enzyme from a cat's tongue perfused for 23 min and activated by infusion of 50 μg ACh. The perfusate was collected in 5 min periods except for the 3 min sample collected during and in the 2 min following ACh infusion.

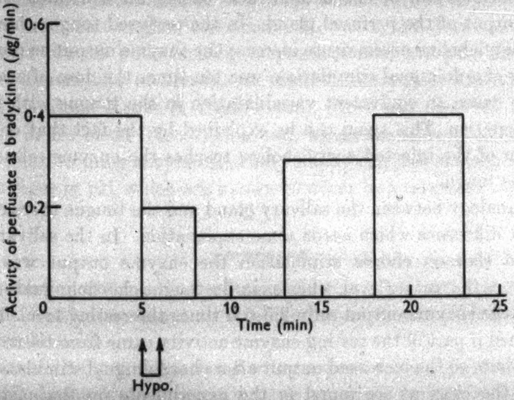

Fig. 5. Output of plasma kinin-forming enzyme from a cat's tongue perfused for 23 min, and activated by 1 min hypoglossal stimulation. The perfusate was collected in 5 min periods except for the 3 min sample collected during and in the 2 min following hypoglossal stimulation.

activity could be detected following a good tetanus of the muscle, produced by stimulation of the tibial nerve.

Skin. In a few experiments in which the blood flow through the skin of the cat's thigh was recorded it was possible to obtain a small antidromic vasodilatation due to stimulation of the saphenous nerve. When the skin was perfused, a small resting output of plasma kinin-forming enzyme was observed, but there was no dectectable increase on stimulation of the saphenous nerve.

DISCUSSION

The finding that stimulation of the chordolingual nerve leads to an escape of the plasma kinin-forming enzyme from the perfused tongue indicates that plasma kinin is formed in the tissue spaces of this organ following such nerve stimulation during normal blood circulation, and that the vasodilatation observed is due in large part to this mechanism. The analogy with the mechanism of functional vasodilatation in the submandibular salivary gland is suggestive, and since hypoglossal stimulation was ineffective in producing an escape of enzyme it is probable that in the tongue the enzyme comes, not from the muscle fibres, but from the glandular elements that form a small proportion of the organ.

The amounts of enzyme escaping into the effluent of the tongue at rest are smaller than the amounts found in the effluent of the perfused salivary gland. This is not surprising if the source of the enzyme in the tongue is the gland cells, because they form only a small proportion of this organ.

In the salivary gland with its normal blood circulation the dose of ACh which produces a vasodilatation of the same extent as that caused by supramaximal stimulation of the chorda, also causes an equivalent increase in enzyme output of the perfused gland. In the perfused tongue, however, the dose of acetylcholine necessary to increase the enzyme output as much as that caused by chordolingual stimulation, was ten times the dose of acetylcholine needed to cause an equivalent vasodilatation in the tongue with its normal blood circulation. This again can be explained by the fact that only a small proportion of the injected acetylcholine reaches the enzyme-releasing gland cells of the tongue.

In the analogy between the salivary gland and the tongue there remains an important difference which needs some explanation. In the salivary gland it was found that on chorda stimulation the enzyme output was increased 2·5–8·5 times the resting level, whereas in the tongue chordolingual stimulation increased the enzyme output only 2·5–5·5 times the resting level. This would be explained if part of the resting enzyme activity came from tissue which did not contribute to the increased output after chordolingual stimulation. This is probably the case, as we found in the experiments on the gastrocnemius muscle that some enzyme activity appeared in the perfusate when the muscle was at rest. It can be concluded that in the perfusion experiments on the tongue it is not only the gland cells which contribute to the resting level of enzyme activity, whereas the increased activity after chordolingual stimulation comes from the glandular tissue alone.

According to Bain (1936) removal of the mucous lining of the tongue did not abolish the vasodilator effect of chordolingual stimulation. A detailed account of this work has not been published, so we do not know if the vasodilatation was reduced by this operation and, if so, how much it was reduced. However, if our conclusion that the enzyme mainly responsible for the vasodilatation in the tongue originates in gland cells is correct, then these glands must be at least partially dispersed throughout the tongue. It is not known whether the islets of salivary gland tissue usually embedded in the tongue are supplied by fibres of the chorda tympani to this organ, but our experiments suggest that the fibres responsible for the vasodilatation in the tongue are secretomotor fibres of the cranial parasympathetic outflow, and not specific vasodilator nerve fibres.

Since the increase in enzyme output on chordolingual stimulation is abolished by hexamethonium, there is no evidence that the small antidromic vasodilatation obtainable in the cat's tongue under these conditions is due to plasma kinin formation. This particular vasodilator reaction is probably produced in some other way, for similar negative results were obtained in the cat's skin, in the territory supplied by the saphenous nerve, where a greater antidromic vasodilatation was obtainable.

Failure to find any increase in enzyme output following tetanic contraction of striated muscle, in the case of both the tongue and the gastrocnemius, indicates that the mechanism of functional vasodilatation in skeletal muscle is different from that in glandular tissue. However, this does not necessarily mean that a plasma kinin-forming mechanism does not play some part in functional hyperaemia in muscle. A possible mechanism has already been suggested by Frey, Kraut & Werle (1950). They had observed an enzyme circulating in the blood (blood kallikrein), which has since been shown to be a plasma kinin-forming enzyme, and they found that this enzyme is activated by the changes in pH which are known to occur in a tetanized muscle.

SUMMARY

1. Stimulation of the cat's chordolingual nerve produces a vasodilatation in the tongue which is resistant to atropine but is almost abolished by hexamethonium bromide.

2. When the tongue was perfused with oxygenated Locke's solution a small amount of plasma kinin-forming enzyme appeared in the venous effluent. The amount did not increase with time of perfusion.

3. Stimulation of the chordolingual nerve while the tongue was being perfused led to a 2·5–5·5-fold increase in the enzyme activity of the venous effluent. It is suggested that this increase is produced by secretomotor fibres to the glandular elements of the tongue, and that their activation leads to vasodilatation as a result of plasma kinin formation within the organ.

4. Antidromic stimulation of sensory fibres in the chordolingual did not contribute to the increased output of plasma kinin-forming enzyme because, after hexamethonium bromide, stimulation of the chordolingual nerve no longer caused an increase in the enzyme activity of the perfusate.

5. Consistent with this finding, antidromic stimulation of sensory nerves in skin did not cause a detectable increase in enzyme output.

6. Although stimulation of the hypoglossal nerve produced a pronounced vasodilatation in the tongue with normal blood circulation, in the perfused tongue stimulation of the hypoglossal did not cause an increased output of plasma kinin-forming enzyme. Similarly, in another striated muscle, the gastrocnemius, no increased output of enzyme followed tetanic stimulation.

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THE EFFECT OF SOME RESPIRATORY MANŒUVRES ON THE HEART RATE

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That there is some form of interaction between respiration and heart rate (H.R.) is very old knowledge, but it was only in the third decade of this century that Heymans (1929), using his technique of the isolated head, studied this interaction, with particular reference to the respiratory arrhythmia.

Sinus arrhythmia becomes very evident when the depth and frequency of respiration are increased as in recovery after exercise. Considering the possibility of sudden large changes in blood flow through the chest producing an alteration of the H.R., some respiratory manœuvres, designed to vary the blood flow through the intrathoracic pressure, have been worked through systematically. But the sudden variations in the H.R. could not have been readily studied on account of the difficulties involved in accurate recording. A new technique has, in fact, made it possible to demonstrate a delay of about 5 sec in the cessation and reappearance of sinus arrhythmia when the breath is held.

Analysis of the results seems to show a causal relationship between some alterations in intrapulmonary pressure and changes in H.R. Explanation of the results seems to involve the arterial baroreceptors or, at any rate, the left side of the heart, as the site of origin of the stimulus, which appears to be change of blood flow. The same mechanism might be involved also during ordinary quiet respiration.

METHODS

The human experiments were performed on a group of fifteen male subjects ranging from 18 to 31 years of age. The subjects practised in advance the type of experiment to be performed, but the timing of events was given by the experimenter.

The H.R. was computed from the QRS complexes of the e.c.g., recorded between the left leg and the forehead. A cardiotachometer of suitable design (Manzotti, 1956*a*) displayed the heart rate on linear ordinates, beat by beat, with a constant delay of 10 m-sec. The accuracy was ±2%. A dotted record was obtained, each dot corresponding to one beat.

In some experiments the respiratory movements were stethographically recorded by tying round the chest a corrugated rubber tube with one end closed and the other end connected through a small-diameter pressure tube to the pressure recorder described by Manzotti (1956*b*, *c*). The same mechanical-electrical transducer was used to record respiratory pressures in those experiments in which the H.R. was correlated with respiratory pressures.

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The animal experiments were performed on cats and rats fully anaesthetized with Veterinary Nembutal (pentobarbitone; Abbott Laboratories) at a dose of 0·5 and 0·9 ml./kg body weight respectively. In cat experiments heparin was also given intravenously (5 mg/kg body weight). Each animal was fitted with a tracheal T cannula. The intrapulmonary pressure was increased by closing one end of the tracheal cannula and connecting the other end to an air reservoir of about 25 l. capacity, where the pressure had been previously raised to the appropriate value. Negative intrapulmonary pressures were obtained by substituting for the positive-pressure reservoir a suction pump and a water valve. The blood pressure in cats was recorded either from the axillary or from the femoral artery. The H.R. in the animals was calculated directly from the e.c.g. record.

RESULTS

The influence of quiet respiration on the heart rate

The H.R. in a normal subject is by no means a steady parameter. Some fluctuations are certainly to be ascribed to the respiration, as is seen in Fig. 1 by comparing the H.R. record with the stethographic record of respiration.

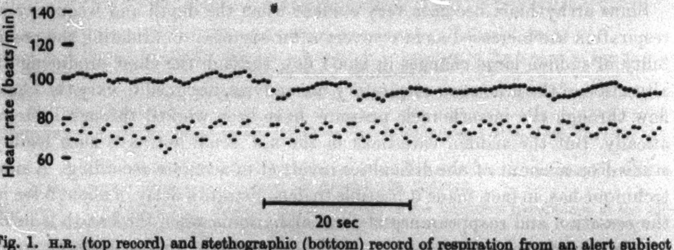

Fig. 1. H.R. (top record) and stethographic (bottom) record of respiration from an alert subject standing and quiet. H.R. in beats/min; inspiration downwards. Some synchronism between H.R. and respiratory cycles is evident (sinus arrhythmia) but is masked by the presence of H.R. fluctuations of non-identified origin.

However, much greater fluctuations occur for apparently no identifiable reason, or if there is any, they cannot be constantly reproduced. This 'background noise' varies from subject to subject, and in the same subject from time to time, but never disappears in the quiet and alert subject.

The occurrence of sinus arrhythmia during recovery from exercise

A closer correlation between respiration and H.R. is seen only under conditions of augmented depth and rate of respiration; the 'background noise' disappears and the respiratory fluctuations of the H.R. become more marked. This is shown in Fig. 2, recorded during the recovery period following mild exercise. The H.R. tracing is to be compared with the stethographic record of respiration.

The effect of some respiratory manœuvres on heart rate

In breath-holding experiments carried out in quiet normal conditions, Fig. 3, 'background noise' as well as sinus arrhythmia disappear. However, the sinus arrhythmia does not stop immediately at the beginning of the apnoeic period, but shows a further diphasic fluctuation. This delayed behaviour is repeated at the end of the breath-holding period, when the sinus arrhythmia sets in only after the full breath following the apnoea. Such experimental evidence suggests the remarkable conclusion that sinus arrhythmia may be a full respiratory cycle behind the respiration.

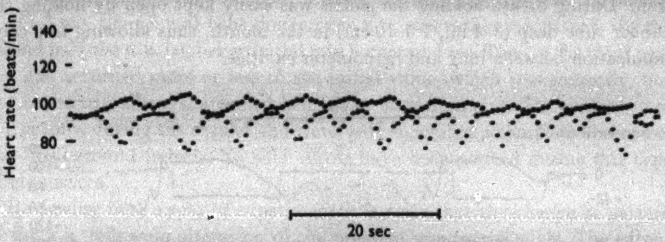

Fig. 2. H.R. (top record) and stethographic (bottom) record of respiration from a subject at rest, recovering from mild exercise. H.R. in beats/min; inspiration downwards. Sinus arrhythmia is more marked than in Fig. 1, and the fluctuations in H.R. of non-identified origin have disappeared.

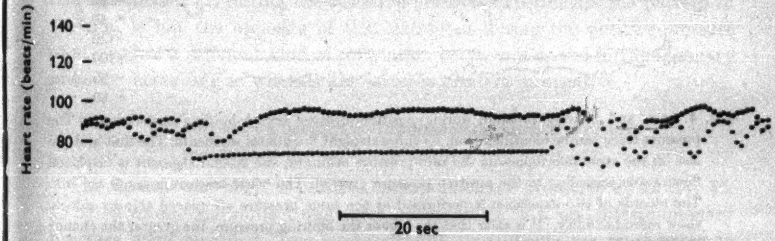

Fig. 3. H.R. (top record) and stethographic (bottom) record of respiration from a subject at rest during a breath-holding experiment. The apnoeic period is signalled by the disappearance of respiratory fluctuations on the respiration trace. Note that during this period sinus arrhythmia disappears as well, but with a delay of about one full respiratory cycle, and with the same delay comes back on resumption of normal breathing. During breath-holding there is no evidence of 'background noise'.

The effect of voluntarily exerted static intrapulmonary pressures upon heart rate

Breath-holding can be performed with either positive or negative pressure in the air cavity of the thorax. For such experiments the subject, while quietly breathing through the nose, held in his mouth a rubber tube connected with a small volume represented by the pressure transducer and by a water manometer. Suddenly, by blowing or sucking through the mouth tube, he produced a predetermined positive or negative pressure that he could read on the water manometer and hold as constant as possible for a period of 20–30 sec. Care was taken that the switching from normal breathing to pressurized breath-holding was not preceded by a deep inspiration or expiration. During breath-holding the glottis was easily kept open by holding the rubber tube deep (3–4 in., 7·5–10 cm) in the mouth, thus allowing free communication between lung and manometer cavities.

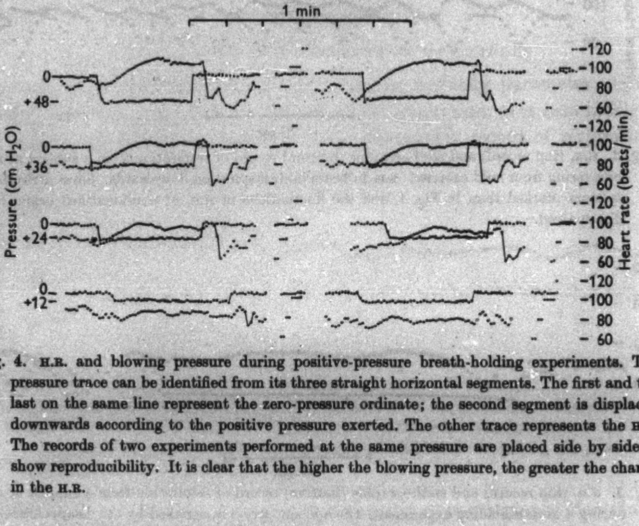

Fig. 4. H.R. and blowing pressure during positive-pressure breath-holding experiments. The pressure trace can be identified from its three straight horizontal segments. The first and the last on the same line represent the zero-pressure ordinate; the second segment is displaced downwards according to the positive pressure exerted. The other trace represents the H.R. The records of two experiments performed at the same pressure are placed side by side to show reproducibility. It is clear that the higher the blowing pressure, the greater the change in the H.R.

The typical behaviour of the H.R. during positive and negative pressure breath-holding is shown in Figs. 4 and 7. The analysis of such responses can be better performed by dividing them into three parts.

Positive-pressure breath-holding. In the first part, starting with the beginning of the pressure exertion and lasting for about 4 sec, the H.R. behaved rather inconstantly, sometimes dropping, sometimes increasing, mostly being constant or slightly increasing. During the second part, that follows immediately and lasts for 6–12 sec, the H.R. constantly increased, in some cases more steeply than in others, the rate of increase diminishing towards the end of this part and becoming zero at the beginning of the following part. Finally, during the third part the H.R., having reached a maximum level, remained at that level for the rest of the breath-holding period with practically no interference from what has been previously described as 'background noise'.

Side effects, constantly present during this manœuvre, were a decrease in amplitude of the radial pulse and an increase in venous pressure, shown by a gradual distension, appearing between the 4th and 6th second, of the veins, proceeding upwards from the neck to the forehead.

Negative-pressure breath-holding. During the first part (see Fig. 7), lasting about 3 sec, the general behaviour of the H.R. was rather inconstant. In the second part the H.R. fell below initial rate for about 4 sec. Then in the third part the H.R. returned more or less to the initial value, which was generally, but not necessarily always, attained before the breaking point. The constancy of the pattern during the second and third part suggested a complete absence of the 'background noise'. No side effects have been noticed during this type of manœuvre.

Reviewing both types of experiment it is clear that the change in pattern of the H.R. following alteration of the pressure was reproducible. The extent of the change in H.R. appeared to depend on the pressure applied, suggesting a definite correlation. However, the facts that (1) the complexity of the two fundamental patterns does not support the possibility of a simple type of correlation and (2) during the negative-pressure experiments the pattern of the H.R. is not the opposite of that exhibited during the positive-pressure ones, suggest a different kind of correlation between H.R. and intrapulmonary pressure, according to whether the latter is positive or negative.

The correlation between intrapulmonary positive pressure, maintained during breath-holding, and heart rate

The breath was held at positive intrapulmonary pressures of 12, 24, 36, 48 cm of water. The subject was quiet and standing. H.R. and intrapulmonary pressure were recorded. Two such series of experiments are represented in Fig. 4 to show reproducibility of the results. Five experiments were performed at each value of pressure.

In general, the higher the intrapulmonary pressure, the higher the maximum value reached by the H.R. The average readings of these maximum values, as calculated for each group of five experiments performed under the same conditions, were plotted against the corresponding pressures, as is shown in Fig. 5 for three subjects. It appears evident that during pressure breath-holding a direct linear correlation exists between the intrapulmonary positive pressure and the maximum value reached by the H.R. The straight line of this correlation extrapolated towards the zero pressure ordinate intersects it at the value to be expected by averaging the values of the H.R. found in all experiments just before the breath is held, that is the initial value.

In other words, calling $R_{\text{max.}}$ the maximum value of the H.R. (beats/min), R_I the initial value in the same units and P the pressure of water (cm) we have the relation

$$R_{\text{max.}} = R_I + aP; \tag{1}$$

where a, a constant in the considered range of pressures, is the measure of the H.R. increase per unit pressure. In the three subjects considered the values for a are: 1·45, 0·661 and 0·595 beats/min/cm H_2O.

A further step towards the elucidation of the above-mentioned correlation is made by analysing the time course of the variation of the H.R. during a single breath-holding experiment (Manzotti, 1956*d*). If r represents the actual H.R. (always in beats/min) and t the time in minutes reckoned from the beginning of the exertion of pressure, it is found (Fig. 6) that $\log_{10} \dfrac{R_{\text{max.}} - R_I}{R_{\text{max.}} - r}$ is a linear function of the square of the time, or

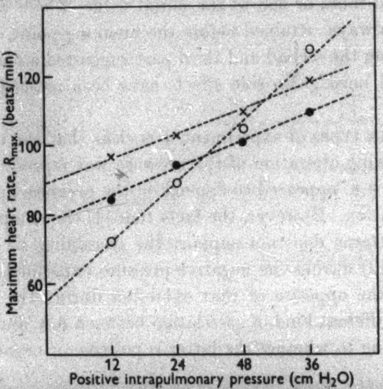

Fig. 5. Relation between the maximum value to which the H.R. is displaced during positive-pressure breath-holding and the blowing pressure. Ordinate, H.R. in beats/min: abscissa, positive pressure in cm of water. The three lines refer to three different subjects. The linearity of the relationship between displacement of H.R. and blowing pressure is evident.

$$\mathrm{d}r/\mathrm{d}t = (R_{\text{max.}} - r)\, tk, \tag{2}$$

where k is a constant with the dimensions of the reciprocal of a squared time and of the value of $1/0{\cdot}012\ \text{min}^2$. Relation (2) has been found consistently constant in many experiments, leaving therefore little doubt about its capacity to represent the experimental results.

The continuity of the time course of the variation of the H.R. as expressed by equation (2) does not support the possibility that the pressure exerted is the direct mechanism that causes such variation. In fact the H.R. varies when the pressure is constant and does not show any discontinuity, as it should if the sudden exertion of producing intrathoracic pressure had any direct effect on it. More direct correlation should be sought amongst those physiological systems that, owing to the pressure, are displaced to a new equilibrium but undergo this displacement slowly and continuously, such as the circulatory system.

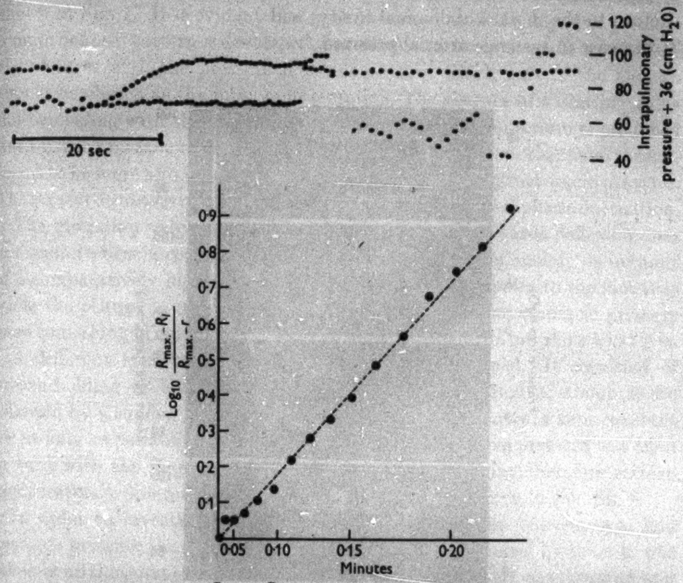

Fig. 6. Relation between $\log_{10}\frac{R_{\text{max.}}-R_l}{R_{\text{max.}}-r}$ (ordinate) and the square of the time in minutes (abscissa) derived from an experiment in which the breath was held at a positive pressure of 36 cm of water (log./log. scale). The linearity of the relation proves the validity of the equation $dr/dt=(R_{\text{max.}}-r)tk$ to represent the time course of the variation of the H.R. induced by positive-pressure breath-holding. Inset: the actual record of the experiment with the same legend as for Fig. 4; for further details see text. ($R_{\text{max.}}=94{\cdot}15$ beats/min; $R_l=65{\cdot}7$ beats/min; $k=1/0{\cdot}012\ \text{min}^2$).

The voluntary effort of producing the positive intrathoracic pressure, which is mainly due to the contraction of the abdominal muscles, brings about a corresponding increase of the intra-abdominal pressure. The circulatory system is, in these conditions, in part—head and limbs—at atmospheric pressure, in part—thoraco-abdominal cavity—at a pressure greater than atmospheric. A new equilibrium, as compared to normal, sets in and is characterized by:

(1) displacement of circulating blood which accumulates in those regions that are at a lower pressure;
(2) decrease in blood flow due to the pressure gradient opposing the venous return to the thoraco-abdominal cavity; and
(3) decrease in systemic arterial pressure.

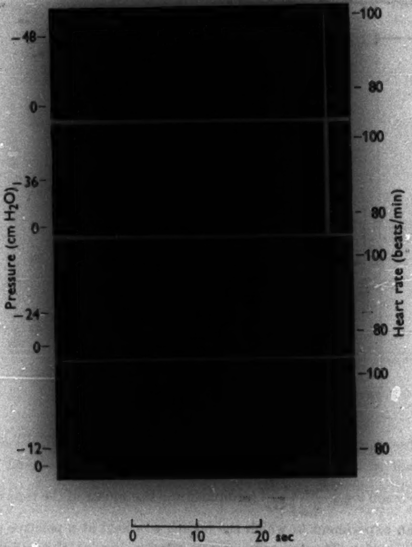

Fig. 7. H.R. and sucking pressure during negative-pressure breath-holding experiments. Same experimental technique as in Fig. 4. The pressure trace is identified in the same way, the segment corresponding to the negative-pressure exertion being now displaced upwards with respect to the zero pressure line. The other trace represents the H.R. The values of sucking pressure in each experiment are, from top to bottom: –48, –36, –24, –12 cm of water. At the right side of each record is the heart rate calibration in steps of 20 beats/min. The typical pattern of the H.R. variation becomes more evident with the increase in negative pressure.

The attainment of this new equilibrium is comparatively slow because of the limited rate at which volumes of blood move from one region to another; therefore it could be considered as directly responsible for the instantaneous behaviour of the H.R. However, the question as to which of the three abovementioned points is the direct cause remains to be answered.

Does a correlation exist between intrapulmonary negative pressure and heart rate during breath-holding?

The typical behaviour of the H.R. in a series of breath-holding experiments performed at intrathoracic pressures of −12, −24, −36, −48 cm of water is shown in Fig. 7. It is evident that with the increase of the negative pressure a more definite pattern is developed, but when an attempt is made to correlate the changes with the pressure it is not obvious which part of the pattern should be chosen as the most representative. The absence of a limiting value not coinciding with the initial suggests that, if a new equilibrium is produced during such a manœuvre, the H.R. is not involved in it. On the other hand the H.R. seems to be more affected by the sudden decrease of the intrathoracic pressure. The greater decreases in magnitude produce a more evident diphasic fluctuation.

The minimum value touched by the H.R. in its downwards deflexion does not seem to bear a correlation with the negative pressure, mainly on account of a comparatively high variability. These observations point to the fact that while the change in H.R. and the exertion of negative intrathoracic pressure have something in common, the experiments performed do not show what it is.

A different type of experiment was therefore designed. It consisted of breath-holding at constant positive pressure for a period of about 20 sec followed by a sudden switching to a negative pressure that was kept constant for as long as possible. Each of the four values of positive pressure was tried in turn with the four values of negative pressures, giving therefore sixteen quantitatively different experiments. The results are shown in Fig. 8*a*, *b*.

In order to investigate the possibility of a correlation between H.R. and negative pressure the average of the minimum values reached by the H.R. was taken in all those experiments, in which the negative pressure was the same, and plotted against this negative pressure. The graph that is obtained, Fig. 9, does not give any support to the idea of there being a possible correlation. However, when those experiments in which the exerted positive pressure was the same were grouped together and the average of the minimum value reached by the H.R. during the phase of negative pressure exertion was calculated and plotted against the positive pressure, Fig. 10, the existence of a correlation appeared evident. These experiments support the view that the decrease in the H.R., following either the release of a voluntarily held positive intrathoracic pressure, or the switching from it to a negative pressure, is related to the total changes that have been previously brought about by the exertion of the positive pressure.

If further analysis of Fig. 10 is carried out, it can be found that

$$dR_{\text{min.}}/dP = (R_0 - R_{\text{min.}})h, \tag{3}$$

where $R_{\text{min.}}$ is the minimum value in beats/min reached by the H.R. after

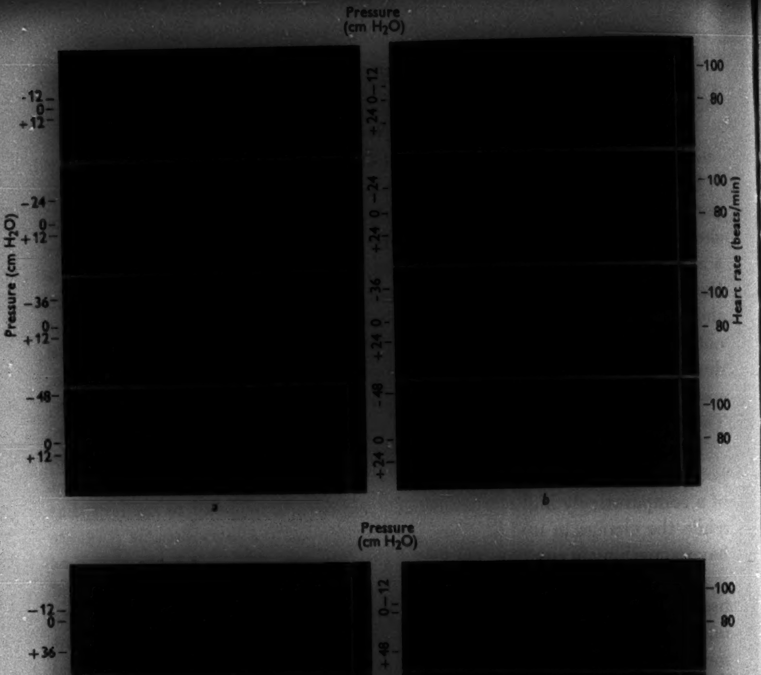

Fig. 8. Representations of the H.R. and pressure records of a group of experiments in which there was an initial phase of about 20 sec of breath-holding at positive pressure (downwards displacement of the manometer trace). The same value of blowing pressure was followed in turn with four values of sucking pressure corresponding to -12, -24, -36, -48 cm of water, and the records of these four experiments are shown in a column from top to bottom. Four values of blowing pressure were used for these, namely $+12$, $+24$, $+36$, $+48$ cm of water and the results for these values appear in columns (*a*), (*b*), (*c*) and (*d*) respectively. Thus the records appearing vertically below each other have identical blowing pressures and those in each horizontal row the same sucking pressure. For identification of pressure and H.R. trace see legends of Figs. 4 and 7. At the right side of each record the heart rate calibration in steps of 20 beats/min.

release of the positive intrapulmonary pressure, R_0 is that minimum value of H.R. (beats/min) that could be attained after exertion of an extraordinarily high positive intrapulmonary pressure, P is the positive pressure in cm of water and h is a constant with the dimension of the reciprocal of a pressure. In the subject considered, $R_0 = 59{\cdot}20$ beats/min and $h = 1/16{\cdot}5$ cm of water.

Relation (3) is supported by the following considerations:

1. The extrapolation towards the left of the line identified by the four points on the graph of Fig. 10 meets the zero pressure ordinate at a point corresponding to the initial value of H.R., that is 77·8.

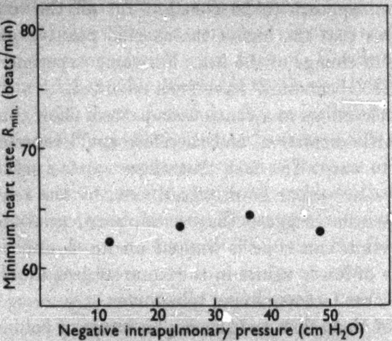

Fig. 9. The minimum value reached by the H.R. during the sucking phase is averaged for the four experiments in each row of Fig. 8, and plotted against the negative pressure exerted. Ordinate, H.R.; abscissa, negative pressure. The lack of correlation between the two parameters is evident.

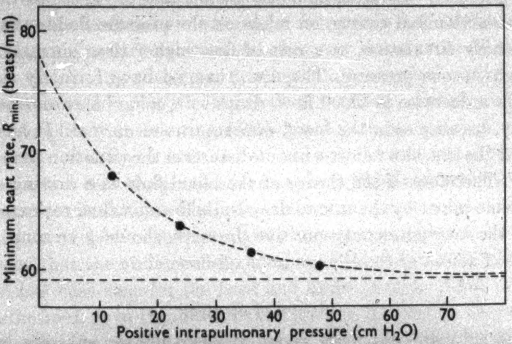

Fig. 10. The minimum value reached by the H.R. during the sucking phase is averaged for the four experiments shown in each column of Fig. 8 and plotted against the positive pressure exerted before the sucking phase. Ordinate, H.R.; abscissa, pressure. An exponential relation between the two parameters is evident.

2. The extrapolation towards right approaches a limiting minimum value of H.R. corresponding to R_0.
3. A straight line is obtained when $\log(R_{\text{min.}} - R_0)$ is plotted against the pressure.

From the experiments of Fig. 8 it is also seen that the H.R., during the negative phase of pressure exertion, varies continuously, and therefore blocked conduction along the specific myocardium can be excluded. This has also been controlled by direct e.c.g. analysis. Moreover, the time taken by the H.R. to drop to the corresponding minimum value, once the negative pressure has been switched on, appears to be constant for all the experiments, about 6·2 sec. This shows that the higher the exerted positive pressure the larger must be the rate of change of the H.R. For some experiments instantaneous decelerations of 2400 beats/min^2 have been recorded.

Such rapid decelerations in a continuous pattern show that the H.R. should reproduce with little distortion, and therefore could represent, the situation that compels it to vary. The fact that there exists a minimum value, R_0, below which the H.R. cannot be displaced even by the release of very high intrapulmonary pressure suggests that the range of variations with which the normal sinus rhythm can cope is limited on its slower side and that the possible existence of lower values in the same subject should be regarded as an indication of other factors having taken over.

The variation of blood flow and the displacement of volumes of blood from one region to another have been mentioned previously as possible causes for the instantaneous H.R. behaviour; this also appears to supply an explanation for the findings of Fig. 8. In fact, the blood that during the positive pressure effort has gradually accumulated on the venous side of the circulation, outside the thoraco-abdominal cavity, on releasing the pressure floods the heart and the pulmonary circulation, at a rate of flow higher than normal on account of the high venous pressure. The H.R., that we have formerly supposed to increase for a decrease in blood flow, drops to a value below normal but only transiently, because soon the blood flow returns to normal. It has also been shown that the H.R. can follow without distortion the situation that may cause it to vary. Therefore, if the theory of the blood flow as a driving stimulus is true, the time taken by the H.R. to drop to its lowest value, reckoned from the release of the intrapulmonary positive pressure, should give some indication of the site of action of the change in blood flow.

Action of atropine

Atropine sulphate has been given to some of our subjects in doses of 1/100 gr. (0·6 mg) by intramuscular injection. No variation of the pattern of response of the H.R. has been detected, in spite of the presence of the other signs of atropinization. The same conclusion, that, at non-toxic doses atropine does not interfere with the H.R. in experiments of this type, can be drawn from the results reported by Lee, Matthews & Sharpey-Schafer (1954) in which the subjects 'received 1 mg atropine sulphate intravenously'.

Animal experiments

An attempt was made to reproduce in animals the same type of H.R. response. The intrapulmonary pressure was suddenly increased or decreased, either by blowing into the lungs or sucking from them, and held constant for 20–30 sec. In the anaesthetized cat (6 animals) there has never been an increase in the H.R. during the positive-pressure manœuvre, even though the rate was reasonably low. The H.R. remained constant or slightly decreased. This finding is in agreement with what Sarnoff, Hardenbergh & Whittenberger (1948), Bjurstedt (1953), Bjurstedt & Hesser (1953) and Bjurstedt, Wood & Åström (1953) have found in the anaesthetized dog. However, Hamilton, Woodbury & Vogt (1939) seem to have been able to record from the unanaesthetized dog a H.R. response to passive increase of the intrathoracic pressure (cf. Figs. 8 and 9 of their paper) comparable to that obtained in man during positive-pressure breath-holding. The arterial blood pressure constantly showed an immediate drop to as low as 30–40 mm Hg for applied intrapulmonary pressures ranging from +10 to +40 cm of water. In man, when the intrapulmonary pressure is voluntarily raised, such a considerable initial drop of the arterial blood pressure is never present (Lee *et al.* (1954); Judson, Hatcher & Wilkins (1955)). During application to our cats of negative intrapulmonary pressure alone no variation of the H.R. was found. When blowing into the lungs was immediately followed by sucking, the H.R. showed an inconstant transient decrease during the sucking phase.

In the anaesthetized rat (six animals) a decrease in H.R. was always present when the intrapulmonary pressure was passively raised to 20–30 cm of water. Frequently the slowing of the H.R. developed into a typical cardiac block such as can be obtained by vagal electrical stimulation. Cutting of both vagi abolished completely the slowing of the H.R.

The divergence between man and animal responses finds a probable explanation in the fact that the passive increase in intrapulmonary pressure blocks the pulmonary circulation. The intra-abdominal pressure remains at about atmospheric level, because of the relaxed state of the abdominal wall, with the consequence that the abdomen becomes one of the regions in which blood is pooled, as has been seen for the head and limbs of man. When the animal is not anaesthetized, as in Hamilton's experiments, the passive distension of the dog lungs probably produces an expiratory effort sustained by a contraction of the abdominal muscles with consequent increase of the intra-abdominal pressure. In this situation, very similar to that in the human, the H.R. seems to show the same type of variation as in man.

DISCUSSION

It is generally assumed that sinus arrhythmia may be due (*a*) to afferent impulses from the lungs, (*b*) to variations in pressure on the venous side of the heart, or (*c*) to some central influence defined as 'irradiation of impulses from the respiratory to the cardiac centre' (Heymans, 1929).

The above experiments show that in the human these views may not be tenable. In fact, it has been seen that on bringing the respiration to a standstill by holding the breath the disappearance of the sinus arrhythmia is delayed about 5 sec and that about the same time elapses before it reappears on resumption of normal breathing. It has also been shown that the heart rate is capable of very high rates of variation not due to extrasystolic or block phenomena. It follows then, that the H.R. can respond with fidelity to those bodily situations that act on its driving mechanism, and that the above-mentioned delay, should views (*a*) and (*c*) be true, ought to be attributed to a nervous mechanism connecting respiration and H.R.

A delay of 5 sec between respiratory and cardiac centres is too long for (*c*) to be a satisfactory explanation. It is also too long to justify assumption (*a*), that lung stretch receptors reflexly drive the H.R. Adrian (1933) and Pitts (1942) have shown that, in the afferent path of pulmonary reflexes, if delay exists it is well below 1 sec. The most probable cause of the sinus arrhythmia is to be found, according to our experiments, in the variations of blood flow and blood distribution brought about by the variations of intrapulmonary and intra-abdominal pressures during respiratory manœuvres.

When pressure is exerted upon the blood vessels two different situations are originated according to whether the pressure acts on the lesser circulation only—increase in intrathoracic pressure by blowing into the animal (Bjurstedt, 1953)—or on the lesser and part of the general circulation—increase in intrathoracic and intra-abdominal pressures during voluntary positive pressure exertion (Wood & Lambert, 1952).

In the first case the blood flow is suddenly stopped and arterial blood pressure falls considerably. Relatively low values of positive intrathoracic pressure can completely block the blood flow on account of the fact that they act against a blood pressure of only 25–35 cm of water in the lesser circulation.

In the second case the situation of the circulation is altered by the presence of an obstacle impairing the venous return to the heart. The blood slowly, according to the rate of flow, pools in those regions, head and limbs, in which the pressure is not exerted. The blood pressure on the venous side of these regions gradually increases until it is able to overcome the resistance offered by the exerted pressure and again to push some blood to the heart, though at a reduced rate of flow.

The view that during respiratory manœuvres the H.R. is strictly connected with these alterations of blood flow or with the consequent variations of blood pressure at the level of some specialized structures with baroceptor activity, is supported by the following findings:

(1) delayed response of the H.R. as compared to that of the respiration;

(2) gradual increase of the H.R. during positive-pressure breath-holding, referable to the slow alterations in the circulation;

(3) attainment during the same conditions of a limiting value of H.R. comparable to the new equilibrium in the circulation, and proportional to the pressure exerted—the decrease in blood flow is believed to be linearly related to the pressure exerted;

(4) no alterations of the H.R. during negative pressure breath holding, that could be correlated with the negative pressure. (No appreciable alteration of the blood flow is in fact expected on account of the fact that the great veins are functionally collapsible, but very little distensible; Otis, Rahn & Fenn (1946).);

(5) transient decrease of the H.R. after release of positive-pressure exertion or after switching from positive- to negative-pressure exertion, which causes a transient increase in venous blood flow to the heart comparable to what would be obtained after removal of an obstacle on the venous return;

(6) correlation of the minimum value reached by the H.R. on suction immediately after release of positive-pressure exertion with the positive pressure itself;

(7) inability to reproduce the type of human H.R. response in anaesthetized animals, where, in spite of a similar increase of the intrapulmonary pressure, the circulatory situation was entirely different.

In conclusion, the sinus arrhythmia can be said to be correlated with the blood flow through the pulmonary circulation in such a way that for a decrease of flow there is an increase in H.R. and vice versa. As regards the location of the receptors driving the H.R. and sensitive either to the blood flow or to some physical features connected with it, it should be noted that the venous side of the heart cannot be taken into consideration, in spite of the generally accepted view (*b*) mentioned on p. 554. In fact the water front of the blood coming from the head and the limbs, on releasing the voluntarily held positive pressure, should take about 6 sec to reach the right auricle. This time is obviously too long. Moreover, the increased distension of the right auricle, generally considered as the basis for the Bainbridge reflex, should bring about an increase of the H.R. This is just the opposite of what happens in our experiments. It is considered that the water front of the blood should go further along the pulmonary circulation and perhaps reach the systemic circulation before the proper baroceptors are stimulated.

It is doubtful whether the same receptors, as far as location is concerned, are responsible for both the H.R. responses, increase during positive pressure effort and decrease after release. For an increase in H.R. is obtained by a moderate decrease of blood flow, as happens in voluntary positive-pressure maintenance. But no increase at all or even a decrease in H.R. occurs on complete arrest of blood flow, as occurred in the anaesthetized animals during passive positive-pressure breathing. An increase in flow had no such effect. It follows then that in those two instances two different types of receptor were stimulated, the one giving an increase, the other a decrease, in H.R. in response to the same stimulus, namely a decrease in flow. There is also the possibility that more powerful reflexes could overrule weaker receptors, and indeed this should be called in to justify the complete absence in our experiments of what is called the Bainbridge reflex.

In spite of these considerations it seems possible to conclude that the drop of the H.R. following release of positive-pressure exertion is brought about by stimulation of the aortic baroceptors due to the transient increase in blood flow and consequently in blood pressure.

SUMMARY

1. The interaction between respiratory manœuvres and heart rate has been studied in man, and in anaesthetized cats and rats.

2. In man sinus arrhythmia has been found to follow the respiratory cycles with a delay of about 5 sec during normal breathing.

3. During voluntary positive-pressure breath-holding, the H.R., initially R_I, slowly reached a maximum limiting value, $R_{\mathrm{max.}}$, related to the pressure (P) by $R_{\mathrm{max.}} = R_I + aP$; a being a constant of the order of 1 beat/min/cm of water. The time course of the variation of the heart rate (r) has been empirically found to be given by $\mathrm{d}r/\mathrm{d}t = (R_{\mathrm{max.}} - r)tk$; the constant k being of the order of 1/0·012 min^2.

4. During voluntary negative-pressure breath-holding no correlation has been found between the pressure and the H.R., in spite of a constant pattern of variation of the H.R. During maintenance of a negative pressure immediately following positive breath-holding, the H.R. dropped to a minimum transient value, $R_{\mathrm{min.}}$, lower than initial, R_I, and related to the immediately preceding positive pressure by the equation $\mathrm{d}R_{\mathrm{min.}}/\mathrm{d}P = (R_0 - R_{\mathrm{min.}})h$; the constant h being of the order of 1/16 cm water; the constant R_0, equal to 59 beats/min, representing the lowest limit to which the H.R. could be displaced.

5. The maximum instantaneous variations of the heart rate not due to extrasystole or heart block have been found to be of the order of 2400 beats/min^2; the H.R. is therefore capable of following without distortion the time course of the events by which its variations are produced during the performance of respiratory manœuvres.

6. In anaesthetized cats and rats, where the intrapulmonary pressure has been passively varied, the heart rate pattern of the response was entirely different from that of man.

7. It is considered that in the experiments on man the heart rate varies according to the variations of the blood flow at the level of the pulmonary circulation brought about by the respiratory manœuvres, and it increases for a decrease of flow and vice versa.

8. It is suggested that the aortic baroceptors represent the sensory area for the blood flow–heart rate reflex.

I wish to express my gratitude to Professor H. P. Gilding for the hospitality I received in his Department and for the experimental help and the constructive criticism he gave me at each stage of the work.

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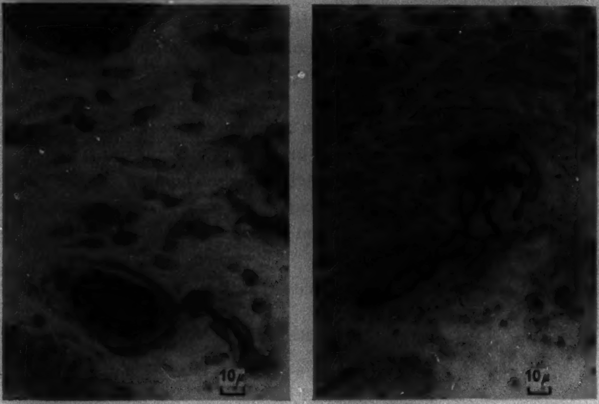
Sensory nerve endings of the anal region in man.

By H. L. Duthie and F. W. Gairns, *Department of Surgery and the Institute of Physiology, University of Glasgow*

The extreme sensitivity of the anal region in man is well known: little detailed work, however, has been done on the microscopic structure of the sensory nerve endings in this region. A systematic histological study of the nerve endings in this region has therefore been undertaken and this is a preliminary report of the findings.



Human anal skin. Bielschowsky–Gros silver diammine ion stain. Untouched photomicrographs.
Fig. 1. Typical Golgi–Mazzoni corpuscle below the epithelium of the circumanal skin near the anal margin.
Fig. 2. Loosely coiled form of ending in a dermal papilla near the anal valves.

Normal perineal skin, circumanal skin and skin from the anal canal with the adjacent rectal mucosa were obtained at three early autopsies and from

a

seven patients at operation. The tissues were fixed in 12% neutral formalin and longitudinal sections, extending from the anal skin to the rectal mucosa, were impregnated by the Bielschowsky–Gros silver diammine ion technique (Garven & Gairns, 1952).

The results are summarized below.

(*a*) The circumanal skin showed numerous typical peritricheal endings and free nerve endings in the stratified squamous epithelium but no organized endings were found.

(*b*) Close to the anal margin the pattern of innervation changed. Hair follicles and their endings became fewer; free nerve endings were still evident though their ramifications in the epithelium were not well shown, owing to the heavy deposits of melanin. Several types of organized nerve endings were here not infrequent; some of these were indistinguishable from Meissner corpuscles, Krause end-bulbs and Golgi–Mazzoni corpuscles.

(*c*) In the anal canal these organized endings became very numerous, six or seven being not uncommon in one low-power microscope field. Here also forms very similar to genital corpuscles were frequent, especially in and around the anal valves and crypts.

(*d*) In the region of the transition from stratified squamous to rectal epithelium, where stretches of stratified columnar epithelium may be found, the free nerve endings were profuse. In addition, many loosely coiled forms of ending were present.

(*e*) In the rectal mucosa proper close to (*d*) only one organized ending has so far been found. In the submucosa and in the muscularis externa, ganglion cells of the plexuses of Meissner and Auerbach were seen along with their processes.

In all regions examined, skin, anal canal and rectal mucosa, nerve plexuses were present in association with blood vessels and the sympathetic ground plexus was also seen.

The expense of the special reagents used was defrayed by the Rankin Fund.

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The assessment of changes in the sensitivity of a stretch reflex.

By T. D. M. Roberts. *Institute of Physiology, University of Glasgow*

There are a great many factors which influence the amount of motor-unit activity evoked by a particular applied stretch. The effective stimulus is the deformation of the receptors, and, for muscle spindles, the effective deformation is presumably related to the tension in the intrafusal muscle fibres. It is not easy to tell what this tension will be for a particular spindle at any particular time.

The total tension in the tendon will be the sum of the tensions in all the active muscle fibres, plus the tensions in the inactive fibres, plus the elastic tension in the non-contractile material which is in parallel. Thus, at a given load, a change in the degree of activity of any single motor unit in the muscle will affect the tension in all the other units. The sensory discharges, from which the reflex motor activity is derived, change with time during steady-states of applied stimulation ('adaptation'). The tension in the elastic materials bears a different relation to length during stretching from that which holds during shortening ('hysteresis').

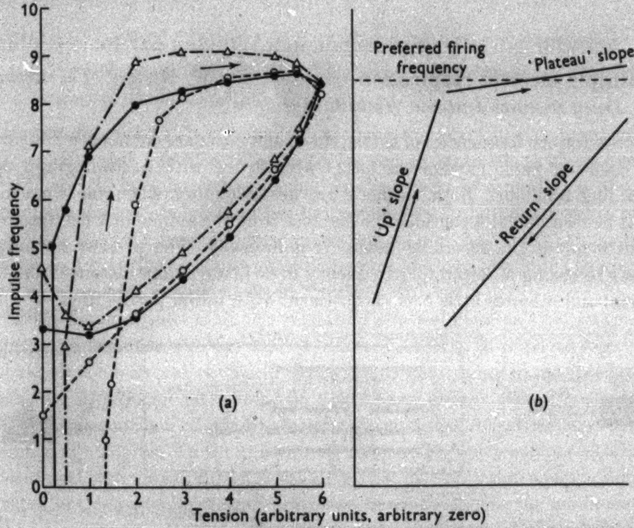


Fig. 1. (*a*) Plot of impulse frequency in soleus motor unit (decerebrate cat) against applied tension. The tension was varied sinusoidally with a period of 3 sec in each case. ●—●, head in normal position; ○--○, head lowered 30°; △--△, head raised 30°. (*b*) Scheme of variables in the observed response. The position and slope of each of the three lines may vary independently.

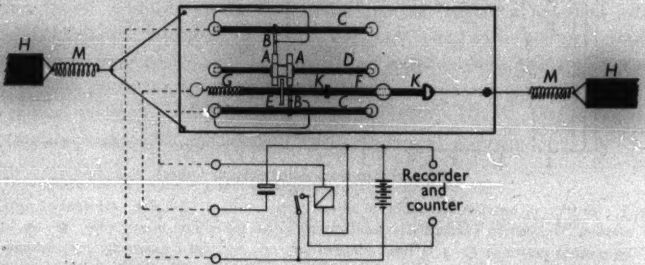
Some clarification can be achieved by applying fluctuating *tensions* in a periodic manner, allowing the muscle freedom to change in length. The responses in successive cycles, as indicated by the impulse frequency of single motor units in the muscle, are sufficiently like one another to permit investigation of the effects of altering other conditions. A study is being made of the effects of change of head position on the time course of the discharge of single motor units in the soleus muscle of the decerebrate cat, during sinusoidal fluctuations of applied tension.

A plot of impulse frequency against applied tension often gives a roughly triangular figure (Fig. 1). The plateau is a common feature. It occurs below the fusion frequency. It is as though, for each motor unit, there is a 'preferred firing frequency'. If a reflex calls for activity in this unit, it fires at its preferred firing frequency, the change from rest to full activity occurring over quite a small tension range. If more tension is required, other units are recruited to share the load. The position and the slope of each of the three lines forming the triangular figure appear to be varied independently.

A simple electric respiration counter.

By W. Nisbet. *The Hannah Dairy Research Institute, Kirkhill, Ayr*

The respiratory frequencies of cattle, sheep and goats are commonly measured by counting flank movements either visually or with a stethograph and recording tambour. Both methods are unsatisfactory when the frequency must be recorded at a considerable distance from the animal or when frequent adjustment of the recording device is undesirable. The present apparatus allows recording of respiratory frequency to be made at any distance from the animal and requires little or no adjustment after initial positioning.



The diagram shows a contact assembly consisting of two brass nuts *A* mounted on an insulating bush. To prevent free movement of the nut assembly, strip springs *B* are soldered to the nuts and rub on rods *C*. 44 s.w.g. copper wire leads couple the nuts to the circuit.

The complete assembly can be moved either way along its slide *D* by the circular brass contact *E* which is soldered to rod *F*. Rod *F* moves back and forth by virtue of its return spring *G* and its coupling to the belt *H* which fastens round the chest of the animal. The respiratory action causes a continual change over of the moving contact with the brass nuts. This switching action will take place anywhere within the limits set by the end stops *K*, which prevent excess movement of the animal damaging the unit while springs *M* take up this unwanted movement.

The unit acts as a single pole change-over switch and it is used to charge a capacitor to a suitable voltage and discharge it through a Siemens H.S. relay driving an electromagnetic counter and pen recorder with inbuilt time marker. On test, the unit was driven at 200 cycles/min and at the end of 2×10^6 counts there was no appreciable wear.

Inhibition of the rabbit colon induced *in vitro* by nicotine. By

J. S. Gillespie and B. R. Mackenna. *Department of Physiology, University of Glasgow*

Nicotine added to the bath usually causes contraction of *in vitro* preparations of the gut: this effect is thought to be due to stimulation of cholinergic ganglion cells in Auerbach's plexus. If the effects of stimulation of these ganglion cells are abolished either by poisoning with botulinum toxin or by atropine, nicotine may produce relaxation (Ambache, 1951; Ambache & Edwards, 1951). This relaxing effect of nicotine has been attributed to stimulation of adrenergic ganglion cells in Auerbach's plexus.

In the ileum of the rabbit this inhibitory effect of nicotine after atropine is difficult to demonstrate (Ambache & Edwards, 1951). In previous work with the rabbit colon *in vitro* we found that while concentrations of nicotine greater than 10^{-5} (hydrogen tartrate $\equiv 3{\cdot}26 \times 10^{-6}$ base) always caused contraction, lower concentrations of nicotine consistently caused relaxation even in the absence of atropine. In the presence of atropine high concentrations of nicotine caused obvious relaxation. The ease with which inhibition could be demonstrated in this region of the gut prompted us to investigate the mechanism of this action. We used the *in vitro* innervated preparation of the rabbit colon with stimulating electrodes on the extrinsic parasympathetic (pelvic) and sympathetic (lumbar colonic) nerves (Garry & Gillespie, 1955).

The demonstration is in three parts:

(*a*) The inhibition induced by nicotine in an atropinized preparation of the rabbit colon is compared with the inhibition produced by stimulating the extrinsic sympathetic nerves in the colon. The ability of TM10 (choline 2:6-xylyl ether bromide) to abolish the effect of nerve stimulation without affecting the inhibition produced by nicotine will also be shown. TM10 is known to abolish the effects of adrenergic nerve stimulation without affecting the response of the effector cells to the catechol amines (Bain & Fielden, 1956; Exley, 1957).

(*b*) Graphs are exhibited to show (i) that the motor effect of high concentrations of nicotine on the colon can be converted into an inhibitory action by atropine; (ii) that when hexamethonium is present the effect of nicotine is abolished whether it be a motor or an inhibitor action; (iii) that the relaxation produced by nicotine is greatly reduced, but not completely abolished, in preparations taken from rabbits treated over the previous ten days with small doses of reserpine given by intravenous injection (0·2 mg/kg/day); (iv) that after prolonged stimulation of the extrinsic lumbar outflow the inhibition produced by nicotine is unimpaired.

(*c*) Histological preparations show enterochromaffin cells in the mucosa of the colon of the rabbit, but complete absence elsewhere in the colon of cells showing the chromaffin reaction. However, after removal of the mucosa—and presumably after removal of all chromaffin cells—the atropinized colon still responds by relaxation to nicotine. Incidentally, after removal of the mucosa the colon responds in characteristic fashion to stimulation of the pelvic and of the lumbar colonic nerves.

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A self-contained defocusing pressure transducer. By J. R. Greer. *Department of Midwifery, University of Glasgow**

The pressure transducer shown was designed to meet the need for a simple robust unit for clinical research on respiration physiology. The unit, which is self-powered, is intended for use in the region of 1–100 mm of water, but the system is essentially stable and a similar mains-operated unit, the complete circuit and constructional details of which have already been published (Greer, 1958), has been used in an integrating flowmeter operating on a pressure gradient of less than a millimetre of water.

* This apparatus was developed during a research programme financed by the Scottish Hospital Endowments Research Trust.

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A sensitive self-contained patient-cycled respirator. By J. R. GREER

*Department of Midwifery, University of Glasgow**

A patient-cycled respirator has the advantage over other respirators that the gas is supplied synchronously with the patient's own spontaneous respiratory effort and never in opposition to it. It operates therefore on the principle of 'augmented respiration' and is consequently more efficient (Donald & Young, 1952; Donald & Lord, 1953; Donald, 1954).

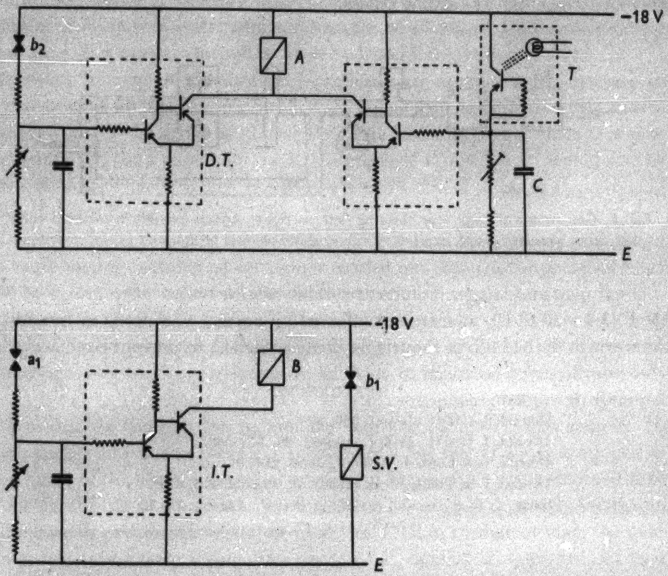

Fig. 1. Electronic circuit of respirator showing: (top) delay timer and trigger unit; (bottom) inspiration timer and solenoid valve. The phototransistor is OCP 71, the other transistors are V 10/15 A.

The respirator to be shown has been completely redesigned from our earlier models to render it self-contained and readily portable. This has been achieved by the use of plug-in transistor units (shown inside dotted lines in Fig. 1). Apart from their inherent compactness, the use of these transistor units greatly reduces the power required, and permits the use of dry batteries. Since a small oxygen cylinder is also included, the respirator can be used

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* This apparatus was developed during a research programme financed by the Scottish Hospital Endowments Research Trust.

for 15–20 min without supplies, the batteries cutting in automatically if the power fails.

The expiratory valve (*E*) and patient-operated trigger (*T*) (Fig. 2) are designed to provide maximum sensitivity with almost unimpeded expiration, the expiratory valve remaining continuously open in the event of failure of gas, while the relief valve (*R*) limits to 30 cm water the maximum pressure that can be applied to the patient. The trigger flap lifts (and blocks the light to the photocell, *P*) for a momentary inspiratory rate of 1 l./min. The capacitor, *C*, prevents triggering by cardiac pulses.

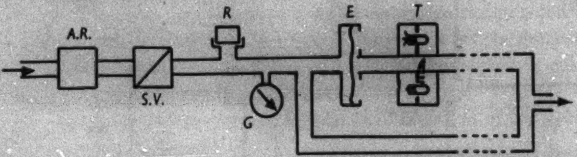

Fig. 2. Gas circuit of respirator showing (left to right) Adams regulator, solenoid valve, relief valve, pressure gauge, expiratory valve, photo-electric trigger and tubing to subject.

The timing and trigger circuits are stable over a temperature range of 35–85° F (1·4 – 29·6° C), and are not affected by normal variations in humidity, the warmth of the bulb in the trigger being sufficient to prevent condensation.

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The dynamics of the frog heart.

By I. A. Boyd and W. R. Eadie. *Institute of Physiology, University of Glasgow and The Boyd Research Institute, Glasgow*

A study has been made of the effect of alteration of venous pressure and of peripheral resistance on the perfused frog heart, using the technique previously described for continuous recording of the heart's activity (Boyd & Eadie, 1957). The venous pressure can be maintained within 0·25 mm of water of any chosen value. A series of fixed 'peripheral resistances' of known value was made, the units of resistance used being those of pressure difference (mm Hg) divided by flow (ml./min), in which case 12·5 such units are equivalent to 10^6 absolute units (dynes. sec. cm^{-5}), the approximate value of the resistance of the systemic vascular bed in a pithed frog. Using any one of these resistances, for streamline flow

$$Q=P_a/R,$$

where Q is the cardiac output, R the resistance, and P_a the mean aortic pressure (the pressure at the other end of the resistance being zero).

To obtain a stable preparation with an arterial pressure close to that of the frog, measured by sphygmomanometry before the experiment began (about 30 mm Hg; Boyd & Mackay, 1957), typical values required are a venous pressure of 15 mm of water and a resistance of 15 units. Under satisfactory conditions, the cardiac output is between 1 and 2 ml./min.

Increase in venous pressure (P_v) produces an exactly linear increase in cardiac output up to a limiting value of P_v above which the heart starts to fail. The gradient of the graph is almost the same for most frogs. Since Q is proportional to P_a for a fixed value of R, the graph of P_a against P_v should also be a straight line. This is, in fact, the case, a rise of 1 mm of water in P_v producing a rise of about 1 mm Hg in P_a.

In some experiments, the heart, liver and lungs have been isolated and placed in a cardiometer chamber. Perfusion is carried out through the posterior vena cava as before, all other vessels, including the pulmonary ones, being ligated. Simultaneous records of cardiac volume, arterial pressure and, in some cases, intraventricular pressure during individual cardiac cycles are obtained on a Cambridge direct-writing recorder, in addition to the usual traces of rate, output, arterial and venous pressures. The intraventricular pressure is obtained with a cannula through the pulmonary vein and collapsed right auricle. If, under these conditions, a negative pressure of a few mm of water is applied to the outside of the heart, an arterial pressure of 30 mm Hg may be obtained with a considerable negative venous pressure, and the output is greatly increased.

Increase in peripheral resistance produces a fall in cardiac output, the relation being very nearly linear. The graph of P_a against R approximates to a parabola, the pressure rising towards a maximum (the maximum height of the fluid column which the heart can support). This peak occurs with a resistance of about 50 units, and the heart starts to fail when the resistance is near this value. The parabolic shape of the curve follows from the observation that the graph of Q against R is a straight line of negative gradient, and the fact that $Q=P/R$. With the perfused frog heart, therefore, the output is not independent of the peripheral resistance, as is accepted generally to be the case in the mammalian heart.

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A methylene-blue technique for staining muscle spindles. By

I. A. Boyd. *Institute of Physiology, University of Glasgow*

Most histological techniques for the staining of nerve endings in muscle have the disadvantage that good impregnation is obtained only occasionally. Experiments are in progress to study the alterations which occur in mammalian spindles after section of spinal roots, and it is obviously desirable to use a staining process which will give good results on every occasion. This cannot be obtained with the gold chloride method previously used in this study (Boyd, 1957). In addition, it was hoped to obtain a technique for making visible the spindles in a living muscle for electrophysiological experiments. A methylene-blue technique seemed to hold the best chance of success.

With very small muscles, immersion in a methylene-blue solution and then exposure to air will produce satisfactory staining. For larger muscles a perfusion method is required; to obtain satisfactory staining of endings deep in the muscle, it is usual to expose the inner parts of the muscle to the air. This results in damage to muscle fibres which then stain blue, obscuring the nerve endings, and, in any case, uniform staining of a whole spindle is rarely obtained.

A method has been developed for staining muscle spindles uniformly while the rest of the muscle fibres are almost completely clear. Successful staining depends on the tissue being in good condition during the process. The muscle is perfused *in situ* with a rapid flow of oxygenated methylene-blue solution of suitable ionic concentration at body temperature. A solution of 0·02 % methylene blue in Krebs's solution with reduced bicarbonate is used (NaCl 128 mM; KCl 5·1 mM; KH_2PO_4 1·3 mM; $NaHCO_3$ 8·6 mM; $CaCl_2$ 2·8 mM; $MgSO_4$ 1·3 mM; glucose 9·8 mM). The solution is continuously aerated with a 95 % O_2 + 5 % CO_2 mixture, and has a pH of 6·6. The abdominal aorta of the cat is cannulated, and all branches except those supplying the lower limbs are tied. Isotonic NaCl at 37° C is perfused through the lower limbs for about 1 min until the fluid coming out of the cut inferior vena cava is quite clear. Then perfusion with the methylene-blue solution is commenced, with the fluid at the tip of the cannula at 39° C, and a pressure head of about 100 mm Hg. The temperature of the emerging solution is about 35° C. After 20 min perfusion the vessels are flushed through again with normal saline for about 1 min. The required muscle is removed and at once transferred to a bath of oxygenated Krebs's solution of the composition given above, at 37° C. The muscle and nerve show almost no trace of blue colour at this point. After about 30 min in the bath, the nerves and motor end-plates on the surface of the muscle stain blue. The deeper nerve branches then start to turn blue, and after about 1 hr the spindles are clearly visible while the rest of the muscle is still almost colourless. The blue colour remains intense for as long as the muscle is kept in the bath. The muscle is fixed in 8 % ammonium molybdate in a cold room during the night. Then it is washed in running water for several hours and finally cleared in glycerine. The spindles remain deeply stained, while the muscle fibres are faintly blue but quite translucent. This technique has produced good results on every occasion. The pH of the solution is important—if higher than about 6·8 extrafusal end-plates and some of the nerves within the muscle will stain but the intrafusal nerve endings hardly stain at all.

The aim of isolating living spindles in good condition by this means has not been attained. When first removed from the animal, the muscle twitches normally when its nerve is stimulated and resting potentials recorded intracellularly are reasonable. When the blue colour begins to appear in the nerves, the muscle twitch becomes less, and, eventually, nerve stimulation produces no response. A similar effect is obtained by intra-arterial injection of a methylene-blue solution into an intact animal.

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An isolated mammalian muscle-spindle preparation.

By I. A. Boyd.
Institute of Physiology, University of Glasgow

Much of the available information about the behaviour of mammalian muscle spindles has been obtained by recording the afferent discharge in sensory nerve fibres from spindles while activating them by stretch or by stimulation of their motor nerves (e.g. Matthews, 1933; Hunt & Kuffler, 1951). The evidence suggests that mammalian spindles contain contracture muscle fibres (i.e. fibres which are capable of maintained contraction, the degree of which depends on the stimulus), but the conception of a spindle as containing contracture fibres only does not fit all the facts. Histological investigation has shown that mammalian spindles contain two types of intrafusal fibre (Boyd, 1957); amphibian spindles contain two types of fibre which are functionally different (Eyezaguirre, 1957). The aim of the present investigation is to isolate mammalian spindles with intact nerve supply, and to determine by direct observation, and by intracellular recording, whether they contain twitch fibres in addition to contracture fibres.

The tenuissimus muscle of the cat was chosen since it can be kept in good condition when isolated, and it contains a row of spindles down the centre. These spindles, however, are not visible in the living muscle, and the problem of locating them before they are damaged in dissection has proved considerable. Practice in the isolation of spindles was obtained by following their large sensory fibres rendered visible in fixed, but unstained, muscles by the use of polarized light, the myelin sheath being birefringent. This technique was less successful in the living muscle, since the muscle fibres are also birefringent.

An attempt was made to obtain a curare concentration in the muscle bath just sufficient to produce neuromuscular block in the extrafusal fibres, in the hope that the spindles might still respond to nerve stimulation (frog intrafusal fibres have a higher curare threshold than the extrafusal fibres; Katz, 1949). It proved too difficult to maintain a critical level of curarization in a bath to which fresh fluid had to be added continuously to keep the muscle in good condition. Next, the spindles in a living muscle were stained with methylene blue (Boyd, 1958*b*), but this also proved unsatisfactory since the muscle then failed to respond to stimulation of its nerve.

In a few cases a successful dissection has been made on the basis of histological knowledge of spindle structure and location. With the rest of the muscle denervated, the isolated spindle is activated by stimulating all the fibres in the whole nerve to the muscle. It has been confirmed by direct observation that the spindles contain contracture fibres. With frequencies of stimulation of about 40/sec contraction of the spindle is just visible; as the frequency is increased the degree of maintained contraction increases until a maximum is reached with a frequency of 80–100/sec. There is an appreciable delay between the onset of stimulation and any visible contraction, and relaxation at the end of stimulation is also delayed. In response to a single stimulus to the nerve certain muscle fibres have been seen to twitch, but it is not yet certain whether these fibres are actually within the spindle or are merely extrafusal fibres closely associated with it and not removed in the dissection. If the latter, then they are innervated by nerve fibres very closely related to those going to the spindle itself, since all other nerve branches have been cut.

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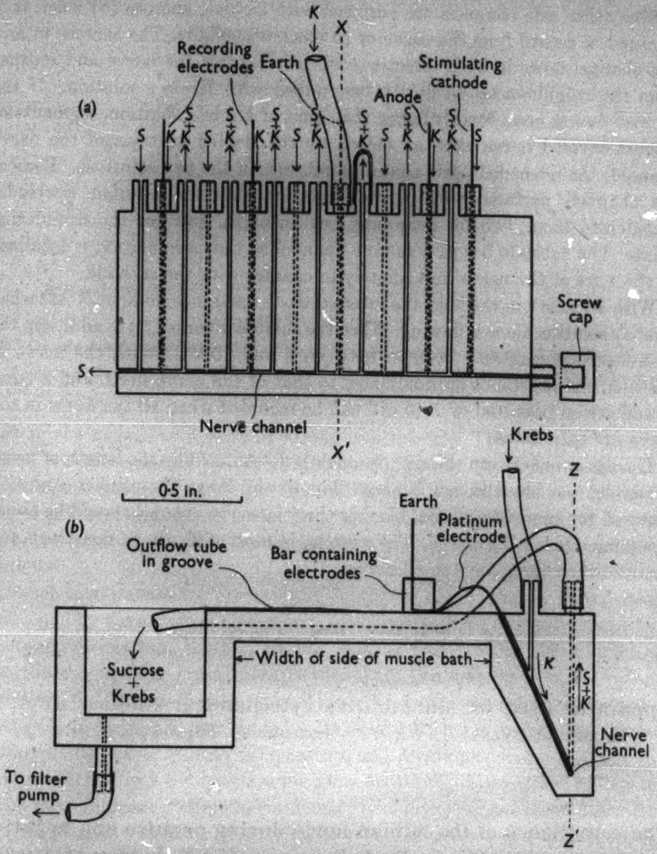
A perfused electrode system for anodal block in a whole nerve.

By I. A. Boyd. *Institute of Physiology, University of Glasgow*

The electrode system has been designed for use with an isolated nerve muscle preparation. The nerve lies in a stream of oxygenated fluid; it is activated at the cathode and impulses in any chosen group of fibres may be blocked at the anode; the compound action potential from the other fibres is recorded so that the blocking process may be accurately controlled. The results obtainable have been described previously (Boyd, 1958).

A solid block of Perspex was milled as shown in Fig. 1. The block fits neatly over the side of the muscle bath and may be moved along the side as required. A very small hole passes through the block from end to end. The nerve is threaded through from one end and secured with the screw cap at the other end.


(a)
K
X
Recording electrodes
Earth
Stimulating cathode
Anode
S
K
S+K
S
K
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S
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S
S+K
S
S+K
K
S+K
S
Screw cap
S←
Nerve channel
X'
Krebs
Z
0·5 in.
(b)
Earth
Platinum electrode
Outflow tube in groove
Bar containing electrodes
Sucrose + Krebs
Width of side of muscle bath
K
S+K
Nerve channel
To filter pump
Z'

Fig. 1. (*a*) Perspex electrode system viewed from the front as though sectioned vertically through the nerve channel (plane *ZZ'* in *b*). (*b*) Vertical section through the earth electrode channel, viewed from the left side (plane *XX'* in *a*). Only one inflow and one outflow tube are shown connected to the block. The tubing connexions are shown as part of the block, though they are made of Perspex rod sealed into the block. Direction of flow of fluid indicated by arrows. *K*, Krebs's solution; *S*, sucrose.

Four side channels are perfused with oxygenated Krebs's solution (K) through separate drop chambers (not shown) to provide electrical isolation. The fluid emerges from a series of outflow tubes, drips into a common chamber, and is sucked away by a filter pump. The outflows, therefore, are electrically isolated from each other.

Five other side channels are perfused with isotonic sucrose (S) when it is required to record from the nerve or to block it anodally. The sucrose in any one channel flows in both directions when it reaches the nerve and escapes from the neighbouring outflow tubes, mixed with Krebs's solution. If the sucrose flow is considerably faster than that of Krebs's solution, segments of sucrose-covered nerve exist as shown by the shaded portions of the nerve channel; the unshaded parts contain a mixture of the two solutions. Four of the channels perfused with Krebs's solution contain platinum electrodes sealed into them, two for recording, one an earth, and one the stimulating anode. The cathode lies in a sucrose channel so that stimulation is localized to the part of the nerve immediately in contact with the cathode.

With no nerve in the hole, the interelectrode resistance is about 50 kΩ when Krebs's solution alone is flowing. When the sucrose is turned on in addition, the interelectrode resistance rises to between 5 and 10 MΩ. With the nerve in position, the resistance approximates to that of the nerve itself, and a compound action potential of 5–10 mV can be recorded when all the fibres in the nerve are stimulated.

The distance between the side channels is determined by the length of nerve available; the block in use is about 2 in. (5 cm) long. The critical condition required for successful anodal block is that the nerve channel should be about the same size as the nerve. The system is most difficult to construct and operate when using very small nerves.

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Apparatus used for quantitative cytochemical studies. By A. J. Hale and Sylvia J. Wilson. *Institute of Physiology, University of Glasgow.*

The compliance of the human lungs during positive and negative pressure breathing. By J. Ernsting. *R.A.F. Institute of Aviation Medicine, Farnborough, Hants*

The volume of blood in the lungs may be altered by changing the pressure in the respiratory tract with respect to the pressure applied to the surface of the body.

The pulmonary compliance (l./cm water) was measured in two seated subjects during positive (up to 80 mm Hg) and negative (up to 25 mm Hg) pressure breathing. The changes in functional residual capacity normally induced by these manœuvres were minimized by applying trunk counter-pressure in the positive-pressure-breathing experiments (Ernsting & Roxburgh, 1957) and by voluntary effort in the negative-pressure-breathing experiments. The pulmonary compliance was determined by recording simultaneously the intraoesophageal pressure and the expiratory pneumotachogram. The change in intraoesophageal pressure and the corresponding tidal volume were determined for six to twelve expirations after 2 min pressure breathing and the compliance calculated.

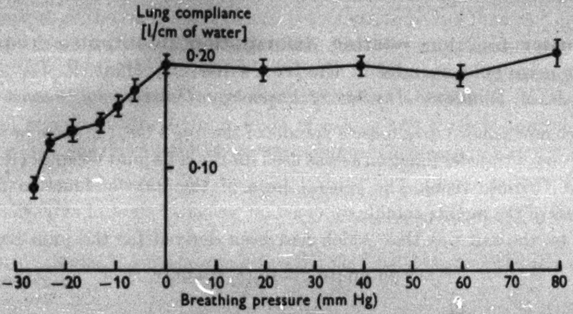

Fig. 1. The effect of the pressure applied to the respiratory tract upon the compliance of the lungs in one subject. Each point and bar represents the mean of ten determinations and ±1 s.e. of the mean.

At each intrapulmonary pressure the volume of the expiratory reserve was determined by the standard spirometric technique and the residual volume by an oxygen rebreathing method (Rahn, Fenn & Otis, 1949). The maximum change in the functional residual capacities of the two subjects from the values when negative-pressure breathing at 25 mm Hg to the values when positive-pressure breathing at 80 mm Hg did not exceed 1 l. B.T.P.S. (volume measured saturated with water at body temperature and pressure).

In neither subject did positive-pressure breathing at pressures of up to 80 mm Hg significantly alter the pulmonary compliance from the resting value (Fig. 1). Negative-pressure breathing, however, caused a marked decrease of the compliance of the lungs of both subjects. The resting pulmonary compliance was halved by negative-pressure breathing at 20–25 mm Hg. Thus in these two subjects a reduction in the pulmonary blood volume did not change the pulmonary compliance whilst an increase in the blood content of the lungs was associated with a marked decrease in compliance.

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A transfer function relating deformation to impulse frequency for a muscle spindle in the frog's toe.

By MARY R. DAVEY and T. D. M. ROBERTS. *Institute of Physiology, University of Glasgow*

We have now applied, to a muscle spindle of the frog's toe, the same procedure for deriving a transfer function as was used for the cat's joint receptor (Roberts, Boyd & Cairnie, 1956). The general form of the transfer function for the responses of the muscle spindle to 'constant velocity' steps of extension turns out to be the same as that which had been derived for the joint receptor, namely

$$F_t = \Psi(s) + \Phi_1(v, t) - \Phi_2(v, t) + \Phi_3(v, t),$$

where $\Phi_i(v, t_1) = \int_{-\infty}^{t_1} \phi_i(v_t)\, e^{-(t_1-t)/\tau}\, \Delta t$, F_t is the impulse frequency at time t, $\Psi(s)$ is the impulse frequency at rest in a position corresponding to the deformation s, $\phi_i(v_t)\, e^{-(t_1-t)/\tau}\, \Delta t$ is a component of the total impulse frequency at time t_1 which is contributed by that particular rate-of-change of deformation v which occurred in the (previous) interval t to $(t+\Delta t)$, and τ is a time constant.

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Changes in the cardiac output of the lamb after birth and during anoxia.

By K. W. CROSS, G. S. DAWES and JOAN C. MOTT. *Nuffield Institute for Medical Research, University of Oxford, and the Department of Physiology, St Mary's Hospital Medical School, London, W.C.2*

It has been realized for many centuries that pulmonary blood flow must increase at birth. Calculation now suggests that in the lamb there may also be a large increase in systemic blood flow. In the lamb towards the end of gestation umbilical blood flow is about 130 ml./kg/min as measured by the venous occlusion plethysmograph or by a flowmeter in the common umbilical vein. Since umbilical flow is on the average 55% of the combined output of *both* ventricles (Dawes, Mott & Widdicombe, 1954) the latter is about 235 ml./kg/min; this agrees well with the cardiometer measurements of Barcroft & Torrens (1946). Of this combined output an average of 12% (say 30 ml./kg/min) goes to the lungs; this figure also is consistent with direct measurements of foetal pulmonary flow. Systemic flow through the tissues of the foetal lamb, other than the lungs, is therefore approximately 235 – 130 – 30 = 75 ml./kg/min. Measurements of right heart output in new-born lambs, 1–60 days old, by the Fick principle, give a mean figure of 325 ml./kg/min. Therefore at birth pulmonary flow increases on the average tenfold, and systemic flow fourfold. The output of the right heart (which is thought to be less than that of the left in the foetus) increases about threefold, the output of the left not quite so much, unless the ductus arteriosus is patent. These calculations may be relevant to the fact that although the adult sheep increases its cardiac output (which is only 100 ml./kg/min while breathing air) three to fourfold during anoxia, the new-born lamb shows no significant increase.

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The characteristics of some points of cardiovascular and respiratory representation in the cerebral cortex of sheep.

By J. F. Mitchell. *A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

During an investigation into the influence of the cerebral cortex on autonomic activity in sheep, a study has been made of the experimental factors which affect the nature of certain autonomic responses to cortical stimulation. From a total of 397 cortical points which yielded cardiovascular or respiratory responses on stimulation, fifty-one have been studied under a variety of experimental conditions.

The anaesthetics used were either cyclopropane or ether. Small alterations in the depth of anaesthesia produced changes in all the responses studied. Anaesthesia was maintained, by reference to the e.e.g., at a level where all somatic motor responses, except those evoked from the low-threshold lip area of the cortex, were abolished. The threshold voltage required to elicit lip movements from the cortex provided an additional guide to the depth of anaesthesia.

The stimulus parameters (see Table 1) influenced both the degree and nature of the responses and each cortical area and each type of response had an optimum set of parameters. Only under these conditions of stimulation were the threshold voltages minimal and the responses from one cortical point reproducible for long periods.

Small variations in the temperature of the cortex (±2° C), which was irrigated at rectal temperature with liquid paraffin, were found to affect both cardiovascular and respiratory responses, whereas larger temperature variations (±5° C) often abolished them. Pressor and depressor responses were found to be maximal at blood-pressure levels greater than 90–100 mm Hg whereas no responses have been observed below pressures of 55 mm Hg.

Table 1. The optimum stimulus parameter ranges for blood-pressure and respiratory responses evoked from three areas of the sheep brain. I, Blood-pressure effects; II, respiratory effects. Lip area of cortex excitable at 3·5–4·5 V. Monitored rectangular pulses from a constant voltage source were used throughout

	Stimulus location	Threshold (V)	Pulse duration (msec)	Pulses/sec
I	Middle longitudinal gyrus	3–6·5	1–5	40–50
	Superior longitudinal gyrus	4–6	5	50
	Orbital gyrus	5–6	10–20	10
II	Middle longitudinal gyrus	5–6	0·1–0·5	50
	Superior longitudinal gyrus	4–5·5	0·1–0·5	50–100
	Orbital gyrus	6–7·5	2–5	50–200

From experiments performed under similar conditions on cats, it appeared that the stimulus thresholds for most responses were lower than in the sheep but that the same factors modified the responses in the same way in the two species.

The ability of the foetal and new-born animal to withstand total anoxia.

By G. S. Dawes, Joan C. Mott and Heather Shelley. *The Nuffield Institute for Medical Research, University of Oxford*

It is well known that immature foetal or new-born animals survive in the absence of O_2 for much longer than adults of the same species. We have extended these observations to the foetal lamb asphyxiated by tying the umbilical cord and preventing inhalation of air.

Foetal lambs of 83–91 days gestational age recovered when the cord was untied after 40 min anoxia; the blood pressure and heart rate, which had fallen during anoxia, returned to their initial levels and there was no evidence of permanent damage to the respiratory centre or other motor centres. Lambs of 120–146 days gestation age could only withstand 15–20 min anoxia without circulatory failure, and adult sheep could not be resuscitated after re-breathing nitrogen from a bag for only 7 min.

During anoxia, large amounts of lactate accumulated in the blood and tissues; this could be accounted for in terms of carbohydrate loss from the blood and heart muscle and, in the older lambs, from the liver. It was observed that the initial carbohydrate (glycogen) concentration in the heart decreased with age and could be related to the survival time during anoxia. This observation was extended to young rats, rabbits and guinea-pigs, since Fazekas, Alexander

& Himwich (1941) had shown that the ability to survive anoxia was very different in these three species. A linear relationship was observed between cardiac carbohydrate and the survival time in nitrogen.

Himwich (1953) and his colleagues concluded that the capacity of any animal to survive anoxia is limited by the ability of the central nervous system to meet its energy requirements by anaerobic glycolysis. Our experiments suggest that the maintenance of an adequate circulation is of the first importance and that this is related to the carbohydrate concentration in the heart at the onset of anoxia.

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Graphic placement of transport segments along the nephron from urine concentration pattern developed with stop-flow technique.

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Our stop-flow technique (Malvin, Wilde & Sullivan, 1957; Wilde & Malvin, 1958) localizes transport across the renal tubular wall from the concentration pattern developed along the nephrons during stopped flow. A polyethylene catheter, reaching into the renal pelvis of a dog under pentobarbital, is clamped for 4–8 min during osmotic diuresis to stop urine flow and filtration. The osmotic diuretic mannitol provides a watery menstruum against which characteristic concentration patterns are developed along the nephrons. On release of occlusion, serial urine samples, delivered in 0·5 ml. portions from the catheter, segment this pattern into an ordered array which is best represented on a graph (Fig. 1) if concentration of each sample is plotted against accumulated volume of urine appearing since reinstatement of flow.

The filtration indicator inulin is injected late after occlusion to reach the filter surfaces and be ready to signal new entry of filtrate into the urine samples after the occlusion is released. The rising concentration of inulin (Fig. 1, right of centre) delineates the new arrival in the samples of inulin from longer and longer nephrons. Paraaminohippurate (PAH), injected before occlusion, reaches its concentration summit in the new outflow pattern in a proximal position just before inulin enters the shortest nephrons.

The blackened segments on the five central nephron diagrams in Fig. 1 indicate the locus of transport segments for PAH and sodium. Their locus was derived from the Na and PAH curves by a system of graphic analysis (Wilde & Malvin, 1958). PAH secretion occurs in proximal segments. Avid sodium reabsorption occurs in distal areas. The strongest potassium reabsorption overlies this distal Na area, but K secretion lies even farther distal.

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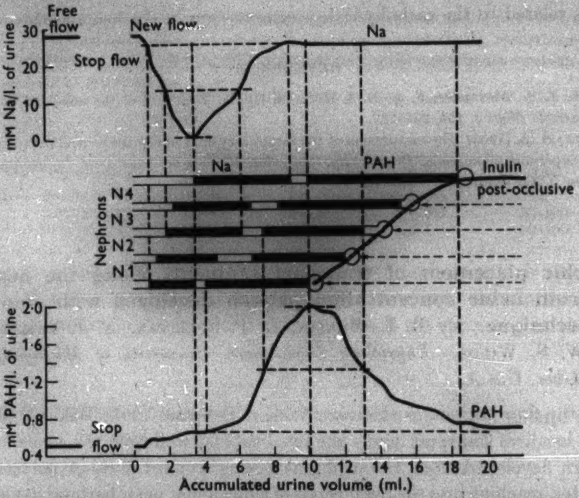

Fig. 1. Graphic placement of transport segments (blackened) for sodium and PAH on to central nephron diagrams from concentration patterns above and below found in the urinary wash-out pattern following stopped flow.

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Potassium transport in seminal vesicle mucosa.

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The seminal vesicle mucosa of the guinea-pig has been shown to retain most of its K during incubation in a Ringer's solution provided glutamate is added and either respiration or anaerobic glycolysis occurs (Breuer & Whittam, 1957).

The tissue resembles frog skin in transporting ions actively in both the presence and absence of oxygen and is different from other mammalian tissues (e.g. kidney, brain, retina, muscle) which require oxygen. Measurements of the easily hydrolysable phosphate (7 min P) of seminal vesicle mucosa have now been made to find if its concentration changed in the same direction as that of K on varying the conditions of incubation. The results show that the labile phosphate (about 8 m-moles P/kg) and the K were maintained both aerobically and anaerobically at 37° C, whereas the addition of 2:4-dinitrophenol (DNP) plus iodoacetate, or a reduction of the temperature to 1° C, caused a fall of 30–60 % in both within 45 min. The rough parallelism between the losses of K and labile phosphate is compatible with a dependence of the active transport of K on phosphate esters from either aerobic or anaerobic metabolism.

The tissue was also incubated in Ringer's solution in which NaCl had been replaced by either choline chloride or sucrose, to see whether the presence of Na was necessary for the retention of tissue K. Partial or complete replacement of NaCl had no significant effect on the tissue K concentration found after 30–60 min aerobic incubation at 37° C (70–85 m-moles/kg), when the tissue Na had fallen to less than 1 m-mole/kg. A reduction of temperature to 1° C, the addition of DNP plus iodoacetate, or the omission of glutamate from the medium, caused a loss of about 20 m-moles K/kg in 1 hr; the addition of 10 mM-glutamate at 37° C after a preliminary period without it caused the uptake of a similar amount of K. The kind of ion balancing the K movement is still unknown. The mechanism for the maintenance of the tissue K concentration therefore seems to be independent of Na movements but dependent on aerobic or anaerobic metabolism.

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The active transport of D-methionine by the intestine *in vitro*.

By E. Lesly Jervis and D. H. Smyth. *Department of Physiology, University of Sheffield*

Wiseman (1953) showed that L-amino acids but not D-amino acids could be moved against a concentration gradient by an *in vitro* intestinal preparation, and he postulated a stereo-chemically specific process concerned with the transport of L-amino acids. Recently, Jervis & Smyth (1958) have shown that competition between D- and L-forms of amino acids for absorption is possible, and this suggests a new interpretation of Wiseman's results. In Wiseman's experiments the amino acids were present initially as a racemic mixture and failure to concentrate D-amino acids might be due to competition of the L-form. Furthermore the movement of water which takes place could prevent increase in concentration of a substance in the serosal fluid unless the substance moved faster than water. Experiments were therefore carried out to determine whether conditions could be found in which movement of D-methionine against a concentration gradient could be shown.

Sacs of everted small intestine of the rat were used as described by Wilson & Wiseman (1954). In order to reduce water transport phosphate saline was used instead of bicarbonate saline and also the ileum was used instead of the jejunum. In each experiment two sacs were made from the distal two-fifths of the intestine below the duodenal-jejunal flexure. D-Methionine was present initially in equal concentration in the mucosal and serosal fluids and its concentration was determined at the end of one hour's incubation in aerobic conditions at 37° C. The typical results of one experiment were as follows. Initial mucosal and serosal concentration = 19·3 mM. Final mucosal concentration = 17·0 mM. Final serosal concentration = 29·1 mM. The initial mucosal volume was 15·0 ml., the initial serosal volume 1 ml., and during the experiment 0·9 ml. of fluid was transferred from the mucosal to the serosal side. The changes in concentration of D-methionine are therefore not due to water movement. Experiments with inhibitors showed that the movement of D-amino acids against a concentration gradient was prevented by KCN (10^{-2} M) or by dinitrophenol (2×10^{-4} M).

These experiments indicate that the movement of D-methionine cannot be regarded as a purely passive process, and there appears to be a mechanism for the transfer of D-methionine against a concentration gradient. There undoubtedly is a mechanism for transfer of amino acids which shows stereochemical specificity, but this specificity is not absolute, at least in the case of methionine. It is of such a nature that L-methionine is transferred more readily than D-methionine, whereas D-methionine if present alone can be transferred against a concentration gradient.

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Certain aspects of reflex control of the stroke work of the heart.

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The relationship between end-diastolic ventricular filling pressure and external ventricular stroke work has been found to be more susceptible of systematization if a related family of curves is superimposed upon the single curve concept created by Starling (Sarnoff, 1955). Those experiments were, in general, aimed simply at amplifying understanding of the performance characteristics of the heart as a pump. The experiments reported herein resulted from attempts: (1) to learn if efferent neuronal pathways can manipulate the ventricular function curve (relationship between filling pressure and stroke work) from right to left (low to high) and vice versa; (2) to ascertain whether baroreceptor stimulation can reflexly control such efferent manipulation; and (3) further to systematize the observed phenomena so as more intimately to relate the performance characteristics of the heart as a pump to the integrated requirements of circulatory regulation.

In dogs under morphine-chloralose-urethane, pressures were recorded in right auricle, pulmonary artery, left auricle, aorta and carotid arteries. Cardiac output and heart rate were also continuously recorded, the latter being controlled by atrial stimulation. Graded stimulation of the left stellate ganglion isolated from the C.N.S. was done after vagotomy. In addition to the above, after arterial isolation of the head, the common carotid arteries were independently perfused at various pressures.

It was observed that: (1) Stimulation of the left stellate ganglion shifted the ventricular function curve upward and to the left and could produce a tenfold augmentation of left ventricular stroke work at the same filling pressure and heart rate. (2) This response was a function of stimulation frequency and voltage. (3) A comparable family of function curves was obtained by varying carotid sinus pressure. These reflex myocardial effects of changing carotid sinus pressures were substantially reduced by isolation of the left stellate ganglion from the C.N.S. (4) Changing from pulsatile to non-pulsatile carotid artery pressures with the same mean pressures had a reflex influence on the position of the ventricular function curve as well as on peripheral vascular resistance. (5) The myocardial effects of stellate stimulation and carotid sinus pressure alteration could be closely mimicked with noradrenaline. (6) At a constant heart rate, a lowering of carotid sinus pressure produced an increase in left ventricular stroke work (at the same filling pressure) which was several orders of magnitude larger than the simultaneously observed increase in peripheral vascular resistance.

It is felt that a possible and, for the time being, more adequate integration of the 'Starling' and 'Reflex' schools of thought might reside in the following generality. At any given level of neurohumoral catechol amine background, an increase in filling pressure will be followed by an increase in the external ventricular stroke work produced and at any given filling pressure an increase in that neurohumoral background will have a similar effect.

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Actions of foetal whale thymus gland extracts on the cat tibialis anterior and sciatic nerve preparation.

By P. T. NOWELL, A. WILSON and H. WILSON. *Department of Pharmacology, University of Liverpool*

Foetal whale thymus glands were extracted with acetone by a modification of the method previously reported (Wilson & Wilson, 1955). The acetone extract was freed from acetone and dried to produce a brown residue. This was dissolved in water and neutralized to yield a clear brown extract (WT1 extract). When WT1 extract was passed through a column of ion-exchange resin (Amberlite IRC50) buffered at pH 6·86 and the column subsequently eluted with 0·2 M-K_2HPO_4, a fraction was obtained which yielded a precipitate with ammonium reineckate. This reineckate complex was converted to a chloride which was a hygroscopic amorphous light yellow residue (product R). The average yield of product R obtained from 1 kg foetal whale thymus glands was 150 mg.

Close arterial injections of product R (0·25–0·38 mg/kg) produced a short lasting potentiation followed by depression of the response of tibialis to single maximal nerve shocks. Potentiation of the twitches was sometimes accompanied by fibrillation of the muscle which lasted about 40 sec. With larger doses (0·5–1·0 mg/kg) a prolonged depression of the twitches rapidly developed and the preparation did not always fully recover 1 hr after the injection. When the twitch height was depressed by product R the effect was not reversed by edrophonium (tensilon) and when a tetanus was interpolated it did not cause further depression of the twitch nor augment subsequent responses to single nerve shocks. No difference was observed in the true cholinesterase activity of homogenates of tibialis taken from the injected and non-injected limbs.

Product R also potentiated the response of the isolated frog rectus preparation to acetylcholine; this effect did not persist after washing with Ringer's solution. These effects of product R, which is free from inorganic cations, are similar to those already observed with WT1 extract and resemble those of a compound with a depolarizing action. The chemical and physical properties of product R are at present under investigation.

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The action of the tracheal pressure on the vascular bed of isolated perfused lungs of cats.

By JEAN BANISTER and R. W. TORRANCE. *University Laboratory of Physiology, Oxford*

The observation of Poiseuille (1855) that inflation of the lung increases the resistance of the pulmonary vascular bed has been supported by most workers, but has recently been questioned by Burton & Patel (1958). They found that inflation of the lung over the physiological range decreased the vascular resistance though this was increased at much greater inflations.

We have made observations at constant tracheal pressures on isolated lungs perfused at steady flows with a plasma-dextran mixture. Between observations the lungs were ventilated with 5% CO_2 in air. In our experiments inflation of the lungs always increased the resistance to flow of perfusate through them. We found that the pulmonary arterial pressure was linearly related to tracheal pressures of 3–16 cm H_2O. With the left atrial pressure kept constant at atmospheric pressure, increases in arterial pressure were approximately equal to increases in tracheal pressure at flows of 30–50 ml./kg/min. Above this range of flows the arterial pressure increased more than the tracheal pressure, but at very low flows it changed less.

These observations suggest that the tracheal pressure affects the vascular bed by pressing directly on some part of it so that a sluice-like action is produced. Some experiments made on the effects of raising the left atrial pressure on the arterial pressure at different tracheal pressures support this view. At a low tracheal pressure raising the venous pressure above atmospheric pressure increased the arterial pressure, but at a high tracheal pressure the venous pressure had to be raised by an amount similar to the increase in the tracheal pressure before the arterial pressure was affected.

A sluice-like action cannot account fully for the observation that at high flows the increase in arterial pressure is greater than the increase in tracheal pressure, nor does it account at all for the small increases in arterial pressure seen on inflation of the lungs when the venous pressure is held at a steady level above the range of tracheal pressures used. It is necessary therefore to postulate some additional action of inflation of the lungs on the pulmonary vascular bed which increases its resistance. This may be a simple lengthening of vessels as the lung expands

If the lungs develop oedema the effect of the tracheal pressure on the arterial pressure is much smaller and flow may occur through the lungs at an arterial pressure less than the tracheal pressure.

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A retinal acuity direction effect. By F. W. Campbell. *Physiological Laboratory, University of Cambridge*

If the pupil of the eye is dilated and a circular aperture of 1 mm diameter is placed close to the cornea, visual acuity is found to be maximal when the aperture is over the approximate centre of the natural pupil. If the aperture is displaced from the centre towards the periphery of the pupil, visual acuity decreases in the meridian at right angles to the meridian of displacement; but it does not decrease in the meridian along the axis of displacement. This effect is best demonstrated by measuring visual acuity with grating test objects. With a 1 mm diameter aperture, visual acuity decreases by a factor of 8 times when the aperture is displaced 4 mm from the centre of the pupil. A logarithmic relation is found between the displacement and the visual acuity.

The effect is not due to spherical aberration, for it occurs when this is corrected with suitable lenses. The same results are obtained when acuity is measured in monochromatic light. A decentred light beam is perceived less brightly (Stiles & Crawford, 1933), but if the luminance of the test object is increased to compensate, or even to over-compensate, for the Stiles-Crawford effect, the loss of visual acuity still occurs.

If the retina is considered to be an infinitesimally thin receptive surface a slight increase, not a decrease, of acuity might be expected with a decentred pupil. An explanation must therefore be sought that involves the optical properties of the central foveal cones, and it should preferably be one that is compatible with an explanation of the Stiles-Crawford effect.

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Pulmonary vasoconstriction in response to sudden forced inflation of the perfused cat lung. By Jean Banister and R. W. Torrance.

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When the tracheal pressure in perfused lungs is increased or decreased within the range 2–20 cm H_2O, the pulmonary arterial pressure changes in the same direction and reaches a steady level in a few seconds. If, however, the lungs are transiently inflated to a tracheal pressure of 40–50 cm H_2O the pulmonary arterial pressure rises abruptly during the inflation and then falls to about its previous level. Over the following half minute, the pressure rises by up to 30 cm H_2O, and then declines to a normal level in the following few minutes. The response cannot be elicited again at full size for about 10 min. The response occurs even if the lungs are held at a steady tracheal pressure before and after the inflation.

The slowness of the rise in arterial pressure could be due either to a slowly developing vasoconstriction, or to a gradual distension of vessels of large capacity proximal to vessels in which constriction was fully established during inflation. The following observations suggest that the first is the correct interpretation.

(1) The pressure rises at a rate which is slow in comparison with the rate at which it changes when the flow into the lungs is suddenly either increased or decreased at the peak of the response or when the outflow from the lungs is completely obstructed.

(2) The time to reach the peak pressure depends little on the rate of flow of perfusate.

The vasoconstriction does not depend on the rise in arterial pressure which occurs during inflation of the lungs: it is seen even if the flow is greatly reduced during the inflation so that the arterial pressure then rises little. Nor is a response produced by obstructing outflow from the lungs until the pulmonary arterial pressure rises to the level seen during inflation of the lungs. In two out of five cases, however, obstruction of outflow until a much higher arterial pressure was reached produced a slight response similar to that seen after inflation of the lungs.

It appears then that inflating the lungs, by stretching some tissue, provokes a constriction of pulmonary vessels, but we have no evidence on which to decide whether the vascular muscle is stimulated directly or some peripheral nervous pathway is involved.

The relationship between 'hunger' and the frequency of occurrence of a response reinforced by food.

By P. B. Dews, *Department of Pharmacology, Harvard Medical School, Boston, Mass., U.S.A.*

Cholinesterase in decentralized and axotomized sympathetic ganglia.

By S. K. Dhar. *Department of Physiology, University College London*

When the post-ganglionic fibres of a sympathetic ganglion of a rat are cut (axotomy) and allowed to degenerate, the ganglion fails to transmit impulses on stimulation of the preganglionic fibres, although the post-ganglionic fibres proximal to the cut still conduct if stimulated directly (Brown & Pascoe, 1952). There is a reduction of the capacity of the superior cervical ganglia of the rats to hydrolyse acetylcholine after axotomy (McLennan, 1954). McLennan did not observe any hydrolysis of benzoylcholine by normal ganglia and concluded that the loss of hydrolytic activity was attributable entirely to a reduction in the true cholinesterase of the ganglion.

I have found significant amounts of pseudo-cholinesterase in the normal ganglia of rats, as judged by the hydrolysis of benzoylcholine. The extent of reduction was investigated separately for true and pseudo-cholinesterases after axotomy and after dividing the preganglionic fibres (decentralization).

Groups of four male rats were used for the estimation of true cholinesterase and groups of six for pseudo-cholinesterase. With aseptic precautions the ganglia were either axotomized or decentralized on the right side, and periods of 3 weeks in the former case and 2 weeks in the latter were allowed for degeneration. The left ganglia served as controls. The ganglia were removed, decapsulated and homogenized in 2 ml. of distilled water. Enzymic activity was estimated manometrically at pH 7·4 and 37° C. The substrates were acetyl-β-methyl choline for true cholinesterase and benzoylcholine for pseudo-cholinesterase in 0·03 M final concentration in small Warburg flasks. Estimations were made in duplicate with controls for non-enzymic hydrolysis, and the results, expressed as μl. CO_2/hr/ganglion, are given in Table 1.

TABLE 1

True cholinesterase	Normal	Operated	% Retention (± S.E.)
Decentralized	42·6	24·4	57·3±3·6
Axotomized	44·5	23·5	52·1±4·7
Pseudo-cholinesterase			
Decentralized	15·2	11·4	75·0±2·9
Axotomized	13·5	11·4	84·4±4·8

It is concluded that both true and pseudo-cholinesterases are present in rats' superior cervical ganglia and that after decentralization or axotomy both are reduced, the reduction of true cholinesterase being much greater than that of pseudo-cholinesterase.

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Single unit activity in the lateral geniculate nucleus of the cat.

By P. O. Bishop, W. Burke and R. Davis. *Department of Physiology, University of Sydney, New South Wales, Australia.*

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